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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING CYTOKINE RELEASE IN RESPONSE TO GENOTOXIC AGENTS

(57) Abstract

A previously unknown mechanism for cytokine release in response to genotoxic agents, as well as therapeutic and diagnostic procedures and techniques which are based on that mechanism, are disclosed. The mechanism involves the recognition of damaged DNA by DNA–protein kinases and the subsequent phosphorylation of substrates that leads to cytokine release. Methods to modulate the activity of DNA–protein kinases and thus the release of cytokines in response to genotoxic agents are described. Assays for DNA–protein kinase activity which can be used to monitor such modulation are also disclosed.

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COMPOSITIONS AND METHODS FOR MODULATING CYTOKINE RELEASE IN RESPONSE TO GENOTOXIC AGENTS

CROSS REFERENCE TO RELATED PROVISIONAL APPLICATION

This application claims the benefit under 35 USC §119(e) of U.S. Provisional Application No. 60/073,640, filed February 4, 1998.

FIELD OF THE INVENTION

This invention relates to cytokines and genotoxic agents. More particularly, the invention relates to compositions and methods which can control, e.g., modulate, the release of cytokines by cells in response to exposure to one or more genotoxic agents.

BACKGROUND OF THE INVENTION

A. Genotoxic Agents

As known in the art, genotoxic agents are those chemicals or treatments, such as heat or radiation, that cause or induce damage to DNA, either directly or indirectly. Such damage can lead to mutations, the stoppage of cell cycling, and/or cell death. The damage may be to the nucleic acid bases or to the sugar-phosphate backbone, or may be single- or double-stranded breaks in the DNA chain. The mutations, when they occur, are heritable changes in the DNA sequence or DNA modification patterns that lead to heritable changes in cell function.

Genotoxic agents are found in the environment as natural components, such as ultraviolet or ionizing radiation, or as natural contaminants in food, such as aflotoxin, or as man-made pollution such as benzo[a]pyrenes in cigarette smoke or industrial emissions. Genotoxic agents are also used for pharmaceutical and health-related purposes. For example, many anti-cancer radiotherapies and chemotherapeutics are

genotoxic agents. Similarly, ultraviolet light, the genotoxic agent to which humans and animals are most often exposed, can be used for beneficial purposes such as tanning. In addition, light or ionizing radiation can be combined with light-sensitizing drugs for dermatological and anti-cancer treatments and to sterilize blood.

One of the biological responses to genotoxic agents is the cessation of cell cycling in order to allow time for DNA repair to be undertaken. Such repair may or may not be successful depending on such factors as the level of DNA repair enzymes within the cell, the extent and type of DNA damage, and the like. The cessation of cell cycling serves the important function of preventing acute damage to the genetic material that would result from cell division without repair. If the damage is irreparable then the cell invokes the apoptosis response, that is, pathways of programmed cell death. This general process of molecular signaling within a cell leading to cessation of cell cycling and/or apoptosis has been recently reviewed by P. Herrlich, C. Blattner, A. Knebel, K. Bender and H. Rahmsdorf, "Nuclear and non-nuclear targets of genotoxic agents in the induction of gene expression. Shared principles in yeast, rodents, man and plants," Biological Chemistry, volume 378, pages 1217-1229, 1997; and J.Y.J. Wang, in "Cellular responses to DNA damage," Current Opinion in Cell Biology, volume 10, pages 240-247, 1998.

B. Long Term Effects of Genotoxic Agents -- Mutations of DNA

The long-term effects of genotoxic agents are mutations in DNA, which occur if DNA repair is unsuccessful. These mutations are heritable changes in the DNA sequence and are an essential element in the process of carcinogenesis in humans. Not all the steps are understood that lead from mutation fixation, that is, permanent establishment of DNA changes, to the development of cancers. It is a characteristic of most human cancers that there is a long, multi-year, latency period between the time of exposure to a genotoxic agent and the development of cancer. This is true despite the fact that the mutations are fixed soon after the genotoxic exposure. Ongoing

changes to tissue containing mutated cells, called tumor promotion, is facilitated by the release of cytokines induced by genotoxic agents and leads to the appearance of tumors.

C. Short Term Effects of Genotoxic Agents -- The Release of Cytokines

One of the important short-term effects of genotoxic agents in animals and man is systemic and involves tissue responses that include erythema, inflammation, fever, antigen-specific immune suppression, and other physiological effects. These effects are mediated by cytokines (for review see T. Luger and T. Schwarz, "Epidermal cell-derived cytokines," in Skin Immune System, ed. J.D. Bos, CRC Press Inc., Boca Raton, Fla., 1990, pp257-291). The present invention is concerned with these cytokine-based responses to genotoxic agents.

As known in the art, cytokines are a large and varied family of proteins that are released by one cell to influence the activity of other cells and/or itself. Cytokine levels are often modulated in response to perturbations of cell functioning and serve to mediate the response and homeostasis of tissues, organ systems, and whole organisms following exposure to genotoxic agents.

Cytokines are not known to be released individually. Rather, they are released as a group that produces a menu of characteristic responses to a genotoxic agent. For example, sunburn caused by solar UV (the genotoxic agent) simultaneously induces: (a) the expression of interleukin-1 (IL-1) that causes fever, (b) interleukin-6 (IL-6) that mobilizes liver function, (c) tumor necrosis factor α (TNFα) that induces inflammation, contributes to local antigen-specific immune suppression and activates latent viruses, (d) interleukin-10 (IL-10) that induces suppresser T-cells, (e) a transient decrease followed by an increase in intercellular adhesion molecule 1 (ICAM-1) that controls infiltration of lymphocytes, and (f) a decline in interferon γ (IFNγ) that modulates immune response, as well as many other cytokines.

In terms of extracellular signaling, the most important cytokines are TNFα and IL-1, since they are capable of not only producing physiological responses themselves but also of inducing the expression of cytokines from other distant cells. Controlling this cytokine-based extracellular signaling caused by genotoxic agents is one of the primary objects of the present invention.

The most common mechanism of action of cytokines is through their release from a cell and migration to another cell. However, in some cases, a cytokine can affect extracellular signaling by being displayed on the surface of the damaged cell. Accordingly, as used herein, the phrases "cytokine release" and "cytokine production" are intended to include extracellular signaling both by actual release of a cytokine from a cell and by extracellular display of a cytokine at the cell surface. These terms are also intended to be interpreted broadly to include any cellular mechanism which results in increased extracellular cytokine signaling, including, without limitation, de novo synthesis, precursor processing, intracellular transport, extracellular discharge, and surface display of a cytokine.

D. Misunderstandings in the Art Regarding the Mechanism of Action of Genotoxic Agents

(1) The Erroneous Strict Dichotomy Theory

To date, those skilled in the art have believed that damage to DNA is responsible for the immediate cessation of cell cycling that can lead to the fixation of mutations in DNA or the apoptotic cell death response following genotoxic exposure. This theory is reviewed by L.H. Hartwell and M.B. Kastan in "Cell cycle control and cancer," Science, volume 266, pages 1821-1828, 1994.

The induction of cytokines, on the other hand, has been thought to occur as a result of damage to the cell membrane, that is, damage to targets removed from the cell nucleus. This theory is reviewed by P. Barnes and M. Karin in "Nuclear factor κB: a pivotal transcription factor in chronic

inflammatory diseases," New England Journal of Medicine, volume 336, pages 1066-1071.

As discussed below, in accordance with the invention, it has been found that this view, i.e., that there is a strict dichotomy between (a) the effects of DNA (nuclear) damage that leads to intracellular signaling and (b) membrane (cytoplasmic) damage that leads to extracellular signaling, is incorrect. In fact, DNA damage caused by genotoxic exposure also leads to extracellular signaling through the production of cytokines.

Moreover, and also in accordance with the invention, it has been found that the production of cytokines by this mechanism requires DNA-protein kinases. As a result, cytokine production arising from genotoxic exposure can be controlled by controlling the levels and/or the activity of DNA-protein kinases. This control mechanism, which is highly effective, has previously been unknown and therefore unutilized.

(2) The Evidence Which Led to the Erroneous Strict Dichotomy Theory

The evidence for the conventional belief that the induction of cytokines is due solely to changes at the cell membrane is very strong. The pathways leading to activation of several proteins capable of activating cytokine gene expression have been established. All these pathways begin with protein kinases that are located at the cell's outer membrane, and most of these protein kinases are related to the inner cytoplasmic portion of a transmembrane protein whose external portion is a cell receptor (reviewed in Y. Devary, R. Gottlieb, T. Smeal and M. Karin, "The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases," Cell, volume 71, pages 1081-1091, 1992).

These protein kinases are activated by events at the cell membrane, distant from the cell nucleus, and activate additional cytoplasmic kinases that culminate in the phosphorylation of systems of gene-activating factors such as AP-1 and NFκB. DNA binding sites for these modified or released gene-activating factors are often found in the promoter regions of genes

coding for cytokines. For example, many of these binding sites are found in the promoter sequence of the TNFα gene, as described by S. Takashiba, L. Shapira, S. Amar, and T. Van Dyke, in "Cloning and characterization of human *TNFα* promoter region," Gene, volume 131, pages 307-308, 1993. It has thus been presumed that the binding of these membrane-activated factors to their binding sites in the promoter regions of the cytokine genes is the pathway by which changes at the cell membrane result in changes in expression of cytokines.

Further evidence for the strict dichotomy theory has come from recent reports which have demonstrated that genotoxic agents such as UV can trimerize cell surface receptors directly and activate kinases that begin cascades presumably leading to signal transduction and gene expression. See I. Warmuth, H. Harth, M. Matsui, N. Wang and V. De Leo, "Ultraviolet radiation induces phosphorylation of the epidermal growth factor receptor," Cancer Research, volume 54, pages 374-376, 1994; and C. Rosette and M. Karin, "Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors," Science, volume 274, pages 1194-1197, 1996.

The strongest evidence, however, has come from the well-studied system of cytokine release following ultraviolet (UV) irradiation. See T. Schwarz and T. Luger, "Effect of UV irradiation on epidermal cell cytokine production," Journal of Photochemistry and Photobiology, B: Biology, volume 4, pages 1-13, 1989. Many cytokines, such as IL-1, IL-6, IL-10, TNF α, ICAM-1 and IFNγ are known to change levels of gene expression (transcription) and release concurrently following exposure of cells to UV.

Using this system, experiments were performed which were thought to (a) definitively demonstrate that gene activation is entirely dependent on events at the membrane and (b) exclude DNA as a target for cytokine gene activation. See Y. Devary, C. Rosette, J. DiDonato and M. Karin, "NFκB activation by ultraviolet light is not dependent on a nuclear signal," Science, volume 261, pages 1442-1445, 1993 and M. Simon, Y. Aragane, A.

Schwarz, T. Luger and T. Schwarz, "UVB light induces nuclear factor κB (NFκB) activity independently from chromosomal DNA damage in cell-free cytosolic extracts" Journal of Investigative Dermatology, volume 102, pages 422-427, 1994.

In some of these widely cited experiments, cells were enucleated (chemically and physically treated to remove the nucleus) and the resulting cytoplasts were irradiated with UV. Despite the absence of nuclei (and genomic DNA), activation of the gene-transcription enhancer NFκB was detected.

Perhaps not surprisingly, these experiments led to the widespread belief among those skilled in the art that the induction of cytokine gene expression following genotoxic treatment is independent of DNA damage and dependent entirely on events at the cell membrane. For example, G. Vile, A. Tanew-Ilitschew and R. Tyrrell summarized the field in "Activation of NFκB in human skin fibroblasts by the oxidative stress generated by UVA radiation", Photochemistry and Photobiology, volume 62, pages 463-468, 1995: "However, UVC radiation-dependent activation of NFκB is evident in enucleated cells and UVB radiation-dependent activation was shown to occur in nuclear-free cell extracts. Thus it appears that, at least with these two agents, the nucleus is not involved in the activation pathway."

There has been some evidence presented that DNA damage may be an initiating event for induction of cytokine gene expression. These reports have shown that the dose-response relation for cytokine gene expression is shifted to lower genotoxic doses in cells that are deficient in DNA repair (see B. Stein, H. Rahmsdorf, A. Steffen, M. Litfin and P. Herrlich, "UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase c-fos and metallothionein," Molecular and Cellular Biology, volume 9, pages 5169-5181, 1989), and that increasing DNA repair by delivery of exogenous DNA repair enzymes reduces cytokine expression or release (D. Yarosh, L. Alas,

J. Kibitel, A. O'Connor, F. Carrier and A. Fornace, "Cyclobutane pyrimidine dimers in UV-DNA induce release of soluble mediators that activate the human immunodeficiency virus promoter," Journal of Investigative Dermatology, volume 100, pages 790-794, 1993).

However, prior to the present invention, there has been no recognized biochemical mechanism by which DNA damage produced by genotoxic agents could be translated into activation of cytokine gene expression, a deficit readily identified by those skilled in the art. For example, Herrlich, Blattner, Knebel, Bender and Rahmsdorf wrote in 1997 in Biological Chemistry, supra, at page 1223: "In conclusion, yeast share a DNA damage dependent pathway of transcriptional regulation with mammalian cells. The components are better characterized due to yeast genetics. The nature of damage recognition and signaling is nevertheless not understood." Similarly, Stephen Jackson wrote in 1997 in "DNA-dependent protein kinase," International Journal of Biochemistry and Cell Biology, volume 29, pages 935-938, at page 937: "Furthermore, by triggering protein kinase phosphorylation cascades, it is possible that DNA-PK activation could induce cellular DNA damage signaling pathways that impinge on the transcription, apoptotic and cell cycle machineries. However, no direct evidence for a role in signaling has so far been demonstrated." Even more recently, Jean Wang wrote in 1998 in Current Opinion in Cell Biology, supra, at page 242: "The UV response can be mediated by the plasma membrane. Whether UV-induced lesions (the cyclobutane dimers and other photoadducts) can generate signals to activate a Rad3/ATM-like protein in mammalian cells is not known." The Herrlich and Rahmsdorf laboratory repeated their 1997 view of the state of the art in 1998 in C. Blattner, Klaus Bender, Peter Herrlich and Hans Rahmsdorf, "Photoproducts in transcriptionally active DNA induce signal transduction to the delayed U.V.-responsive genes for collagenase and metallothionein," Oncogene, volume 16, pages 2827-2834, 2832, 1998: "It is

yet unknown how DNA lesions in transcribed genes are processed to elicit signal transduction."

Without a biochemical mechanism -- the key link -- the data that DNA damage may be an initiating event for induction of cytokine gene expression has largely been ignored. Moreover, without knowledge of a mechanism, control of the mechanism, and thus control of cytokine release, was clearly impossible. The present invention provides these missing elements in the art.

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the invention to provide methods and compositions for controlling (modulating) the release of cytokines upon exposure to genotoxic agents.

A particular object of the invention is to provide such methods and compositions where the genotoxic agent is ultraviolet light, the most common genotoxic agent to which humans and animals are exposed, and the control (modulation) involves reducing the release of cytokines in response to the genotoxic agent.

It is a further particular object of the invention to provide such methods and compositions where the genotoxic agent is a chemotherapy or radiotherapy agent used in cancer treatment and again, the control (modulation) involves reducing the release of cytokines.

It is a further object of the invention to reduce the susceptibility to genotoxic agents (e.g., ultraviolet radiation) of individuals who have had an organ transplant and are receiving immunosuppressive drugs to prevent rejection of the transplant.

It is an additional particular object of the invention to provide such methods and compositions where the genotoxic agent is an immunosuppressive agent, and the control (modulation) involves increasing the release of cytokines in response to the genotoxic agent.

It is an additional object of the invention to identify individuals who need to have the level and/or activity of one or more of their DNA-protein

kinases modulated to modulate the individual's response (sensitivity) to one or more genotoxic agents. In connection with this object, it is a further object of the invention to identify the particular DNA-protein kinase or kinases whose level and/or activity is in need of such modulation.

It is a further object of the invention to provide procedures for determining whether or not an immunosuppressive agent is being administered at a level which will provide a desired reduction or increase in DNA-protein kinase activity sufficient to affect cytokine release in response to genotoxic agents.

It is an additional object of the invention to provide improved assays for levels of DNA-protein kinase activity.

The invention achieves the foregoing and other objects through the discovery that DNA-protein kinases, a class of enzymes which recognize changes, e.g., double-stranded breaks, in DNA and phosphorylate other proteins and/or themselves, play a role in the release of cytokines in response to genotoxic agents. In particular, it has been discovered that one or more DNA-protein kinases are required for transcription and/or translation of cytokine genes after exposure to genotoxic agents.

As used herein, a "DNA-protein kinase" is a member of the family of proteins and protein complexes that respond to changes in DNA structure or conformation by phosphorylating other proteins and/or themselves. The characteristics of this family of enzymes has been reviewed by S. Jin, S. Inoue and D. Weaver in "Functions of the DNA Dependent Protein Kinase," Cancer Surveys, volume 29, pages 221-261, 1997.

Heretofore these enzymes have only been implicated in (1) the formation of immunocompetent cells especially those that require gene rearrangements involving double-stranded DNA breaks, (2) repair of double stranded breaks in DNA, and (3) regulation of cell cycling and the apoptosis response following DNA damage. These connections have been reviewed by M. Hoekstra in "Responses to DNA damage and regulation of cell cycle

checkpoints by the ATM protein kinase family," Current Opinion in Genetics & Development, volume 7, pages 170-175, 1997.

Significantly, with regard to UV light, the most ubiquitous of the genotoxic agents, no member of the family of DNA-protein kinases has been related to UV induced release of cytokines from cells. As noted above, the view of those skilled in the art that cytokine gene expression was controlled by membrane interactions eliminated these DNA-protein kinases from consideration as participants in the signal transduction cascade leading to cytokine release.

For example, in the above cited review, M. Hoekstra wrote on page 170: "In this article, I discuss the PIK kinase (*PI3-kinase-related protein kinase)* family members properties as sensors for cell cycle regulation." In another example, Hosoi et al., in their paper entitled "A phosphotidylinisitol 3-kinase inhibitor wortmannin induces radioresistant DNA synthesis and sensitizes cells to bleomycin and ionizing radiation," International Journal of Cancer, volume 78, pages 642-647, 1998, demonstrated that wortmannin is an inhibitor of DNA-protein kinases but ascribed any effects of wortmannin on cytokines to inhibition of the membrane-bound phosphotidylinisitol 3-kinase (PI-3). At page 645 of their article, these authors wrote: "Wortmannin inhibits cytokine/chemokine-mediated signal transduction pathways by inactivation of PI3-kinase...."

This state of the prior art is represented schematically in Figure 1, where the upper dotted box shows the traditional pathway of damage to DNA leading to activation of DNA-protein kinases, which phosphorylate key protein substrates, which then leads to cessation of cell cycling and apoptosis due to intracellular signaling. Cytokine release, on the other hand, was ascribed in the prior art to the pathway of the lower dotted box, where the genotoxic agent affects the cell membrane and/or cell membrane receptors, which activate a lipid and protein kinase cascade, which then leads to cytokine gene expression and extracellular cytokine release.

Figure 2 schematically shows the genotoxic response pathways in accordance with the invention. A primary difference between Figure 1 and Figure 2 is that the prior art scheme of Figure 1 did not distinguish between early and late events, i.e., events occurring within 3 hours of exposure to a genotoxic agent (early events) and those occurring 6 hours or more after exposure (late events). This time dimension is an important aspect of the discovery which forms the basis of the present invention.

An even more fundamental difference between the pathways of Figure 2 and those of Figure 1 is the fact that Figure 2 includes the production of cytokines as one of the effects arising from damage to DNA by genotoxic agents, such production leading to such biological effects as erythema, antigen-specific immunosuppression, melanogenesis and tanning. This cytokine-production effect of genotoxic agents is shown at the lower right of the right hand box of Figure 2, and as indicated in that box, the effect depends on the action of at least one DNA-protein kinase.

Different types of DNA damage produced by different genotoxic agents activate different types of DNA-protein kinases. For example, double-stranded breaks activate the DNA-protein kinases known generally as DNA-PK and ATM, while UV-induced photoproducts in DNA activate the DNA-protein kinase known generally as FRAP. On-going research in various laboratories throughout the world is expected to identify other forms of DNA damage and other members of the DNA-protein kinase family that fit this pattern and the present invention shall be equally applicable to these subsequent DNA damage/DNA-protein kinase combinations.

As illustrated in Figure 2, the common features upon which the invention is based are that (1) a DNA-protein kinase is central to the recognition of altered DNA and (2) the phosphorylation of downstream proteins results in cytokine gene expression, i.e., cytokine gene transcription and/or translation. Based on this discovery, cytokine production in response to genotoxic agents can be controlled by modulating the levels or activity of one or more DNA-protein kinases.

Such modulation can be achieved by: (1) inhibiting the activity of the one or more DNA-protein kinases using one or more inhibitors, (2) increasing or decreasing the transcription and/or translation of genes involved in the production of the one or more DNA-protein kinases, and/or (3) enhancing the activity of the one or more DNA-protein kinases by, for example, providing the kinase with enhanced levels of the damaged DNA which it responds to and/or with enhanced levels of the substrate which it phosphorylates.

In one specific application of the invention, compounds that inhibit DNA-protein kinases, such as rapamycin, are used to block induction of cytokines by genotoxic agents. Other inhibitors of DNA-protein kinases include pyrophosphate, wortmannin, 6-dimethylaminopurine, the pyridone derivative OK-1035, and single-stranded DNA, as described by S.P. Lees-Miller, "The DNA-dependent protein kinase, DNA-PK: 10 years and no ends in sight," Biochemistry and Cell Biology, volume 74, pages 503-512, 1996.

The exposure to the genotoxic agent may be unintentional, as in exposure to environmental pollution or exposure to sunlight during day-to-day activities. The exposure may also be intentional, and the side-effects undesirable, as in the case of intentional sun tanning or cancer radiotherapy or chemotherapy. Induction of cytokines may be unwanted because they are immunosuppressive, inflammatory, activate viruses, cause unwanted pigmentation, keloids, adhesions or scarring, or other primary or side-effects of exposure to genotoxic agents.

Specific examples of these aspects of the invention include the incorporation of one or more DNA-protein kinase inhibitors, e.g., rapamycin or rapamycin-like compounds, in skincare and suncare cosmetics and pharmaceutical products to prevent unwanted side-effects of sun and pollution damage to DNA, such as erythema, inflammation, immune suppression, activation of latent herpes infections, activation of proteases (e.g., collagenase and metallothionein proteases), and skin cancer.

DNA-protein kinase inhibitors such as rapamycin or rapamycin-like compounds may also be used in combination with cancer chemotherapy drugs or radiotheraphy procedures to reduce the side-effects associated with such treatments, such as, fever, erythema, nausea, vomiting, headaches, chills and abnormal pigmentation. In these applications of the invention, the DNA-protein kinase inhibitor or inhibitors are preferably administered in close temporal proximity to the exposure to the genotoxic agent, such as sunlight, air pollution, chemotherapy, or ionizing radiation, most preferably 30 minutes to one hour prior to exposure.

The invention can also be used to avoid the most deleterious side effects of immunosuppressive therapy in transplantation. Organ transplants have become quite common with the introduction of well-tolerated immunosuppressive compounds such as cyclosporin. However, a major side effect of this immunosuppressive therapy has been a rise in skin cancers on sun exposed skin of these patients. See M. Glover, C. Proby and I. Leigh, "Skin cancer in renal transplant patients," Cancer Bulletin, volume 45, pages 220-224, 1993.

Because the mechanism by which cyclosporin provides immunosuppression is by interfering with calcineurin, it does not block the release of cytokines following UV-B exposure. See A. Marionnet, Y. Chardonnet, J. Viac and D. Schmitt, "Differences in responses of interleukin-1 and tumor necrosis factor α and secretion to cyclosporin-A and ultraviolet B-irradiation by normal and transformed keratinocyte cultures," Experimental Dermatology, volume 6, pages 22-28, 1997. As such, cyclosporin does not have the beneficial effects of a DNA-protein kinase inhibitor, such as rapamycin, in blocking induction of UV-inducible cytokines in sun-exposed skin.

The current invention teaches that genotoxic-exposed organs in general, and sun-exposed skin in particular, should be treated with a DNA-protein kinase inhibitor, such as rapamycin, at a time just prior to or at or following the time of genotoxic exposure, in order to prevent the induction

of cancers caused by the genotoxic agent while maintaining the generalized state of immune suppression required to retain the organ transplant. When the DNA-protein kinase inhibitor is itself an immunosuppressive agent capable of suppressing rejection of a transplant (such as rapamycin), the inhibitor may be used with or without other immunosuppressive agents and/or other types of drugs or treatments. In such cases, the regimen of administration and dosage levels of the immunosuppressive agent are selected to take into account both its ability to suppress transplant rejection and its ability to suppress the production of cytokines in response to genotoxic exposure.

Other uses of the invention include compounds that augment the activity of DNA-protein kinases in order to enhance the induction of cytokines following genotoxic treatment (hereinafter referred to as "DNA-protein kinase enhancers"). Thus some genotoxic treatments are used to induce immunosuppressive responses. For example, psoralen-plus-light is used in skin grafts and psoriasis to induce immunosuppressive cytokines and suppress antigen-specific autoimmune responses. In view of the mechanism set forth in Figure 2, compounds that enhance the activity of DNA-protein kinases can make these immune suppressing therapies more brisk, stronger, and/or more uniform, thus increasing the efficacy of the therapy. Thus, in accordance with the invention, compounds that increase DNA-protein kinase activity are used in conjunction with, or in place of, genotoxic agents to enhance the desired immunosuppressive response.

For example, in accordance with these aspects of the invention, one or more compounds that act like UV-irradiated DNA or short pieces of duplex DNA and thus can stimulate DNA-protein kinase activity are applied at the time of the genotoxic treatment, or in place of the genotoxic treatment, to stimulate the release of immunosuppressive cytokines and provide relief from diseases related to autoimmune responses. Examples of such compounds include lipid or liposome bound duplex DNA and/or its congeners.

Another application of the invention is to identify individuals who need to have the level and/or activity of one or more of their DNA-protein kinases modulated to modulate the individual's response (sensitivity) to one or more genotoxic agents. In accordance with these aspects of the invention, the DNA-protein kinase activities and/or levels of a patient suffering from a disease in which a genotoxic agent produces an insufficient or excessive cytokine expression are screened to identify the specific DNA-protein kinases responsible for the patient's symptoms. For example, some forms of dermatitis, such as atopic dermatitis, lupus erythematosus and porphyria, are caused by immune system over-reaction to environmental UV light or pollution. By screening such patients for DNA-protein kinase activity, those patients who would benefit from inhibitors of specific DNA-protein kinases can be identified. In these applications of the invention, cell extracts are prepared from tissue samples, and a DNA-protein kinase assay is performed.

The assay can, for example, be of the type described below in Example 4, in which antibodies are used to immunoprecipitate the DNA-protein kinase, and then the precipitated DNA-protein kinase is exposed to varying types of DNA damage together with its substrate, such as p53 protein. By measuring the degree of p53 phosphorylation one determines the DNA-protein kinase activity. When compared to normal controls, these studies determine if there is more DNA-protein kinase expressed in the diseased tissue or if the DNA-protein kinase activity is greater in such tissue. This information can then be used in diagnosing disease and selecting therapeutic treatment.

In accordance with various of the foregoing aspects of the invention, assays for DNA-protein kinase levels/activities are required to determine, for example, if a drug is being administered at a level sufficient to inhibit or induce cytokine release. As illustrated in Example 4 below, the invention provides effective assays for this purpose in which: (1) a sample of cells is obtained from the subject, (2) a preparation containing DNA-protein

kinase(s) is obtained from the sample, (3) the preparation is exposed to DNA damage of the type(s) the kinase(s) is (are) sensitive to together with the appropriate substrate(s) for the kinase(s), and (4) the level of substrate(s) phosphorylation is used as a measure of the level/activity of the kinase(s).

The foregoing and other aspects of the invention are discussed in further detail below in connection with the detailed description of the invention and its preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is flow diagram showing the understanding in the prior art of the pathways involved in the response of cells to genotoxic agents. As shown therein, DNA damaged by genotoxic agents was thought to activate DNA-protein kinases that then produced phosphorylated substrates, which then triggered intracellular events of cessation of cell cycling and apoptosis.

Figure 2 is a flow diagram showing the pathways involved in the response of cells to genotoxic agents in accordance with the present invention. As shown therein, DNA damaged by a genotoxic agent activates DNA-protein kinases that then phosphorylate substrates which trigger extracellular release of cytokines.

Figure 3 is a family tree of DNA-protein kinases. The relationships of the known lipid and DNA-protein kinases are shown based on amino acid sequence homologies. The proteins are divided into those with lipid kinase activity and those with protein kinase activity. For each protein the name and the organization of the protein is shown. The thin solid bars indicate regions of non-homology, while the thick solid bars represent regions where small subunits, such as the Ku or FKBP proteins, bind. The open thick bars represent the region of the kinase active site, and the vertically striped region is the carboxy terminus that shows homology among the DNA-protein kinases. For each protein the number of amino acids (where known) and the percent similarity (identical or conserved amino acid substitutions) to the kinase region amino acid sequence of the ATM protein

are shown. This figure is drawn from V. Zakian, "ATM-related genes: what do they tell us about functions of the human genes?" Cell, volume 82, pages 685-687, 1995, and S. Jin, S. Inoue and D. Weaver, "Functions of the DNA Dependent Protein Kinases", Cancer Surveys, volume 29, pages 221-261.

Figure 4 is a Western blot of TNFα protein expression in human keratinocytes. Cells from the human line HaCat were irradiated with 200 J/m^2 UV-B or treated with 1 μg/ml LPS and incubated for 24 hours at 37°C. Extracts were prepared, electrophoresed in a 15% polyacrylamide gel, transferred to nitrocellulose, and blotted with antibodies against human TNFα followed by secondary antibodies linked to horseradish peroxidase and exposed using the ECL chemiluminescence system. The lanes are: (1) irradiated; (2) irradiated and treated with 2 ng/ml rapamycin beginning 30 minutes prior to irradiation; (3) treated with LPS; (4) treated with LPS and rapamycin; (5) authentic TNFα standard.

Figure 5 shows the induction of chloramphenicol acetyltransferase (CAT) from the TNFα promoter following UV exposure in the presence and absence of various DNA-protein kinase inhibitors. XP12BE cells, deficient in nucleotide excision repair, were transfected with the TNFcat transgene to form the XPTNF2 cell line that expresses CAT from the TNFα promoter. The cells were treated with DNA-protein kinase inhibitors beginning 30 minutes prior to irradiation and then exposed to 100 J/m^2 UV-B. After 18 hours, extracts were prepared and assayed for CAT activity using fluorescent chloramphenicol substrate. The reaction products were separated by thin layer chromatography and the fluorescence visualized by UV-A light. The samples are: (1) substrate alone; (2) untreated cells; (3) UV irradiated cells; (4) cells UV irradiated and treated with rapamycin; (5) cells treated with rapamycin alone; (6) untreated cells; (7) UV irradiated cells; (8) cells UV irradiated and treated with wortmannin; (9) untreated cells; (10) UV irradiated cells; (11) cells UV irradiated and treated with staurosporine; (12) LPS treated cells; and (13) cells treated with LPS and rapamycin.

Figure 6 shows induction of chloramphenicol acetyltransferase (CAT) from the TNFα promoter following LPS treatment. XPTNF2 cells were treated as described in Figure 5, except for exposure to 1 μg/ml LPS instead of UV. CAT activity was calculated from the amount of protein extract and the amount of product formed in 30 minutes, quantified by computerized image analysis of the fluorescent thin layer chromatography plate.

Figure 7 is a Western blot of $p70^{S6K}$ phosphorylation in human keratinocytes. Cells from the human line HaCat were irradiated with 200 J/m^2 UV-B or treated with 1 μg/ml LPS and incubated for 24 hours at 37°C. Extracts were prepared, electrophoresed in a 10% polyacrylamide gel, transferred to nitrocellulose and blotted with antibodies against the serine and threonine phosphorylated form of human $p70^{S6K}$ followed by secondary antibodies linked to horseradish peroxidase and exposed using the ECL chemiluminescence system. The lanes are: (1) unirradiated; (2) irradiated; (3) irradiated and treated with 2 ng/ml rapamycin beginning 30 minutes prior to irradiation; (4) treated with LPS; and (5) treated with LPS and rapamycin.

Figure 8 shows FRAP and ATM kinase activity on p53 peptide. Extracts were prepared from the human keratinocyte line HaCat. The extracts were incubated with antibody against FRAP (black bars) or ATM (gray bars) and the bound antibody-kinase product was precipitated with Protein G agarose beads by centrifugation. The bead-bound FRAP kinase was mixed with the FKBP protein and both FRAP and ATM were incubated with a peptide portion of the p53 protein. To this mixture were added various DNAs and inhibitors, as shown. After 2 hours at 30°C, the reaction products were diluted eight-fold, added to ELISA plates and developed with antibodies against phosphoserine-modified protein and phosphothreonine-modified protein, and alkaline phosphatase secondary antibodies with nitrophenyl phosphate substrate. Controls included phosphoserine- and phosphothreonine-bovine serum albumin. Phosphorylated proteins were measured by optical density at 405 nm.

Figure 9 is a dose response curve showing the level of inhibition of TNFcat expression for different concentrations of rapamycin. The CAT activity was calculated from the fraction of acetylated chloramphenicol as described in Figure 5 and compared to the activity in the absence of rapamycin.

The foregoing drawings, which are incorporated in and constitute part of the specification, illustrate various embodiments of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the drawings and the description are explanatory only and are not restrictive of the invention.

DETAILED DESCRIPTION OF THE INVENTION
AND ITS PREFERRED EMBODIMENTS

As discussed above, key aspects of the present invention are: (1) the discovery that DNA-protein kinases play a central role in the release of cytokines by cells in response to genotoxic agents, and (2) the application of that discovery to modulate cytokine release through the administration of DNA-protein kinase inhibitors (if cytokine release is to be reduced) or enhancers (if cytokine release is to be increased).

A. DNA-Protein Kinases

DNA-protein kinases were originally recognized by animal and human mutants that lacked activity in one member of this family of enzymes.

Mice with SCID (severe combined immunodeficiency disease) failed to generate a complete immune system due to failure of immunoglobulin gene rearrangements. These mice were found to have a genetic mutation that inactivated a DNA protein kinase activity essential to a process of immunoglobulin gene rearrangement involving double stranded DNA breaks. In this way, an intermediate in development of immune cells, double-stranded breaks, resembles DNA damage. See the review by S. Jackson, "DNA-dependent protein kinase," in the International Journal of Biochemistry and Cell Biology, volume 29, pages 935-938, 1997.

Patients with the genetic disease AT (ataxia telangiectasia) have loss of muscle control (ataxia), and abnormal blood vessels in the eye (telangiectasia), as well as a predisposition to cancers of the blood such as lymphomas. These patients have an abnormal gene referred to as ATM (AT mutant).

When the SCID and ATM genes, respectively, of these diseases were cloned and their nucleotide sequences analyzed they revealed greatest homology to 3'-phosphotidylinositol (3'-PI) kinases, a family of lipid, not protein, kinases, as reviewed in S. Jackson, supra. This at first was quite puzzling, as no lipid kinase activity could be detected biochemically. It is now recognized that despite the sequence homology with 3'-PI kinases, these enzyme are true protein kinases.

DNA-protein kinases are found in all eucaryotic organisms from yeast to humans. Each cell has more than one type of DNA-protein kinase, drawn from this large family of similar enzymes. The family of amino acid sequence related DNA-protein kinases includes, among others, the original DNA-PK_{cs} and Ku subunits related to the SCID mutation, ATM, ATR, TEL1, MEC1, MEI41, FRAP, TOR1, TOR2, and RAD3, as described in Jin, Inoue and Weaver, 1997, supra. This family of DNA-protein kinases and its sequence homology are shown in Figure 3. Research is on-going to identify additional members of the family.

DNA-protein kinases are in general comprised of two subunits, one much larger than the other. The smaller subunit is not shown in Figure 3. Both the SCID and AT diseases result from mutations in the gene coding for the smaller subunit. These genetic mutants, as well as inhibitors that block DNA-protein kinase activity, have been used to analyze the functions which DNA-protein kinases perform.

B. DNA-Protein Kinase Inhibitors

As discussed above, in accordance with certain of its aspects, the present invention is concerned with compounds that interfere with the activity of one or more DNA-protein kinases, whether by directly

inactivating the active site or sites of the DNA-protein kinase, by directly interfering with binding of the DNA-protein kinase to DNA and/or the substrate or substrates which the DNA-protein kinase phosphorylates, by competing with the DNA-protein kinase for binding to DNA and/or the substrate(s), or by interfering with assembly of the subunits of the DNA-protein kinase.

Examples of DNA-protein kinase inhibitors which can be used in the practice of the invention include rapamycin, pyrophosphate, wortmannin, 6-dimethylaminopurine, OK-1035, staurosporine, and single-stranded DNA. These inhibitors, when combined with suitable vehicles known in the art, can be administered to a subject in various forms, including orally, by injection, and topically. The level of administration will depend on the specific subject, inhibitor, and genotoxic agent, and can be determined in accordance with standard medical practices for therapeutic treatments.

In particular, a level of inhibitor administration is selected which can be tolerated by the patient and which achieves a reduction in DNA-protein kinase activity sufficient to reduce the expression of cytokines in response to one or more genotoxic agents. Cytokine expression levels can be measured using standard techniques known in the art. Suitable inhibitor dose levels and administration regimens can thus be selected by monitoring cytokine expression in response to genotoxic agents as the dose/regimen is varied. For example, levels of TNF-α can be monitored after UV exposure for subjects receiving one or more DNA-protein kinase inhibitors. It should be noted that whereas primary cytokines, like TNF-α increase with exposure to genotoxic agents, secondary cytokines can have more complex kinetics, e.g., levels of interferon-γ can fall and levels of ICAM-1 can fall and then rise in response to a genotoxic agent, such as UV. If such a secondary cytokine is used to determine doses/regimens for a DNA-protein kinase inhibitor, these more complex kinetics need to be taken into account, e.g., a level of inhibitor may be selected which maintains the level of interferon-γ activity at a predetermined value after genotoxic exposure.

In certain preferred embodiments of the invention, the level/regimen of inhibitor administration is selected by assaying for DNA-protein kinase activity, e.g., by assaying for DNA-protein kinase activity using the assays discussed herein. In accordance with these embodiments, a level/regimen of inhibitor administration is selected which produces a substantial decrease (e.g., a decrease of 10%, preferably 50%) in the activity of one or more DNA-protein kinases. For example, levels of FRAP activity can be monitored and selected so as to reduce the release of cytokines upon exposure to one or more genotoxic agents. Again, UV exposure can be the genotoxic agent, with the level of FRAP being selected to minimize TNF-α release.

The DNA-protein kinase inhibitor can be administered either continuously or, preferably, in connection with the exposure of the patient to the genotoxic agent(s). Most preferably, the DNA-protein kinase inhibitor is administered in advance of exposure to the genotoxic agent(s), e.g., 30 minutes before exposure, with the administration being continued through exposure and for a period thereafter, e.g., 24 hours after exposure. Administration at less than before, during, and after exposure can also be used in the practice of the invention, but is less preferred.

C. Rapamycin

A particularly important inhibitor of DNA-protein kinase activity is rapamycin. This inhibitor specifically inhibits the assembly of the large and small subunits of FRAP, as described by E. Brown, P. Beal, C. Keith, J. Chen, T. Shin and S. Schreiber, "Control of p70 S6 kinase by kinase activity of FRAP *in vivo*," Nature, volume 277, pages 441-446, 1995. These authors have identified mutant FRAPs that confer resistance to rapamycin, demonstrating the specificity of the drug.

In contrast to rapamycin, wortmannin, another important inhibitor of DNA-protein kinase activity, blocks FRAP by altering the key amino acids involved in phosphorylation, as described by M. Wymann, G. Bulgarelli-Leva, M. Zvelebil, L. Pirola, B. Vanhaesebroeck, M. Waterfield, and G. Panayotou, "Wortmannin inactivates phosphoinisitide 3-kinase by

covalent modification of lys-802, a residue involved in the phosphate transfer reaction," Molecular and Cellular Biology, volume 16, pages 1722-1733, 1996.

Rapamycin is used clinically today to suppress the immune system during grafting and transplantation because it blocks the proliferation of traditional immune cells, e.g. T cells, as described in M. Suthanthiran and T Strom, "Immunoregulatory drugs: mechanistic basis for use in organ transplantation", Pediatric Nephrology, volume 11, pages 651-657, 1997. It has not been used in conjunction with short term exposure to genotoxic agents, but rather has been used for extended periods in order to retain transplants. Significantly, patients receiving immunosuppressive therapy, including patients receiving rapamycin, are explicitly directed to avoid genotoxic exposure such as sunlight during the entire extended periods of immunosuppressive therapy which can and usually does continue for years. See M. Glover, C. Proby and I. Leigh, "Skin cancer in renal transplant patients", Cancer Bulletin, volume 45, pages 220-224, 1993.

Currently, rapamycin is in the late stages of clinical testing, and is commercially produced by Wyeth-Ayerst Laboratories, a division of American Home Products, as Rapamune® (sirolimus/rapamycin). It is used either alone or in combinations with low doses of cyclosporin A in treatment of transplant patients.

When delivered by the oral route, typical dose ranges for rapamycin are 2 to 5 mg per day. When delivered by the topical route, typical concentrations are in the range of 0.2% w/v. When delivered by the intravenous route, maximally tolerated doses are in the range of 25 mg per kg of body weight. Clinically, doses for immunosuppressive purposes are in the range of 0.5 to 25 mg per square meter of skin surface per day.

In particular, a loading dose of rapamycin of approximating 21 to 24 mg per m^2 body surface area is initially delivered intravenously, as described by C.G. Groth, C. Brattstrom, and L. Backman, "New trails in transplantation: how to exploit the potential of sirolimus in clinical

transplantation", Transplantation Proceedings, volume 30, pages 4064-4065, 1998, and B. Kahan, "Rapamycin: personal algorithms for use based on 250 treated renal allograft recipients", Transplantation Proceedings, volume 30, pages 2185-2188, 1988. This is followed by doses of about 1- 7 mg/m^2 adjusted to yield trough concentrations of 15-30 ng/ml in whole blood for 2-3 months, and then further reduced to achieve trough concentrations of about 10-15 ng/ml in whole blood thereafter. However, because most rapamycin is sequestered in red blood cells, the plasma and tissue concentrations of rapamycin and metabolites are less than 2 ng/ml, as described by D. Trepanier, H. Gallant, D. Legatt and R. Yatscoff, "Rapamycin: distribution, pharmacokinetics and therapeutic range investigations: an update", Clinical Biochemistry, volume 31, page 345–351, 1998.

Significantly, such tissue levels are insufficient to modulate DNA-protein kinase activities after genotoxic exposure and thus modulate cytokine release in response to such exposure. This is because concentrations higher than 2 μg/ml (or about 2 nM) are necessary in order to achieve substantial inactivation of DNA-protein kinases, specifically, FRAP kinase. As shown below in Example 5 below, UV induction of TNFα is insensitive to rapamycin at doses less than 2 μg/ml. Thus, the current practice of administering rapamycin to achieve whole blood levels of 10-30 μg/ml do not achieve rapamycin levels in the plasma or tissues that are sufficient to inactivate FRAP according to the invention.

To date, the recommended doses of rapamycin have been determined solely by the ability to prevent rejection of transplanted organs, and not on the doses required to inhibit FRAP activity in genotoxically-exposed non-immune cells. In addition, the doses are prescribed for extended, long-term use and are not modulated for exposure to genotoxic agents.

In contrast to these previous uses of rapamycin, in accordance with the invention, rapamycin is used to reduce the release of immunosuppressive cytokines, such as, IL-1, IL-6, IL-10, ICAM-1 and

TNFα, through administration immediately before and shortly after exposure to a genotoxic agent, such as UV. Moreover, rapamycin is used at dosage levels high enough to achieve such reduction in the release of cytokines, i.e., at levels higher than those used to prevent transplant rejection. Thus, in accordance with the invention, rapamycin is used to preserve, instead of suppress, the immune system, specifically, to preserve the immune system after genotoxic exposure.

This idea of using an immunosuppressive drug to protect the immune system is clearly counterintuitive and runs opposite to the understanding and practice of the existing art.

D. DNA-Protein Kinase Enhancers

Although the most common applications of the invention will involve reductions in cytokine release, in some cases it is desirable to increase such release. In particular, certain therapies involve using genotoxic treatments to abrogate antigen-specific immune responses (see below).

Enhancers of DNA-protein kinase activity include (1) short segments (e.g., segments having a length of less than about 25 thousand base pairs) of double-stranded DNA, i.e., segments of DNA which have ends to which the DNA-protein kinase can bind, (2) damaged double-stranded DNA which can be short or long strands, (3) DNA-protein kinase subunits in situations where such subunits have been depleted, (4) the substrate or substrates which the DNA-protein kinase phosphorylates, and (5) ATP.

The DNA-protein kinase enhancers can be administered in the same manner as discussed above in connection with DNA-protein kinase inhibitors except that, since the enhancers are biologicals, vehicles/carriers which preserve the enhancer's biological activity and promote its delivery to target tissue should be used. For example, in the case of DNA, cationic lipids can be used to deliver the DNA into cells, and in the case of proteins, topical administration can be performed using a liposome delivery system. See Yarosh, U.S. Patent No. 5,190,762.

Doses/regimens for enhancers can similarly be determined using the procedures discussed above with regard to inhibitors. Thus, the enhancer will normally be administered prior to the exposure to the genotoxic agent, with the administration being continued during the exposure and for a period of time thereafter. Again, monitoring of cytokine and/or DNA-protein kinase levels can be used to determine appropriate doses and regimens for particular subjects, enhancers, and genotoxic agents.

E. Modulation of Cytokine Release

As discussed above, in accordance with the invention, cytokine release following genotoxic exposure can be either decreased or increased by inhibiting or enhancing DNA-protein kinase activity. In most cases, a decrease in cytokine release will be desired, the most common example being reducing cytokine release as a result of UV exposure.

Another example where reduction is desired is in connection with cancer chemotherapy and radiotherapy. Many chemotherapy drugs, such as carmustine and mitomycin C, and many radiotherapies, such as treatments with x-rays, are genotoxic. In accordance with the invention, one or more DNA-protein kinase inhibitors are administered to a patient undergoing such therapy to reduce the side-effects of the therapy.

As with other applications of the invention, the one or more inhibitors are preferably administered in advance of a therapy session, with the administration being continue for a period after the session, e.g., for a day or so. Preferably, the DNA-protein kinase inhibitor or inhibitors are delivered in such a manner that they reach the tissues that have suffered the undesired genotoxic damage from the therapy. In the case of chemotherapy, the DNA-protein kinase inhibitor or inhibitors are typically administered in the same manner as the chemotherapy agent, but they may also be specifically directed to, for example, the gastrointestinal track (oral administration), the scalp (topical administration), and/or the site of injection of the chemotherapy agent (topical or subcutaneous administration) where the side-effects of chemotherapy are most common.

The same types of specific administration can be used in the case of radiotherapy.

Patients undergoing transplant rejection therapy are a particularly in need of protection from genotoxic agents because their immune systems are suppressed. As discussed above, these patients commonly suffer from skin cancer on sun exposed skin, the onset of such cancers often being within a few years of the beginning of therapy. Although DNA-protein kinase inhibitors, specifically, rapamycin, are used in such transplant rejection therapy, these uses do not achieve protection from genotoxic exposure. The present invention teaches that the DNA-protein kinase inhibitors should be delivered in different forms and in different ways from the forms and ways in which they have been used in the transplant field.

Specifically, these inhibitors, such as rapamycin, should be given immediately before and for a short time after genotoxic exposure. In those cases where the systemic use of immunosuppressive drugs is limited by toxicity, additional localized application of DNA-protein kinase inhibitors should be used to alleviate the effects of genotoxic exposure. In particular, in the case of sun exposure, one or more inhibitors should be applied topically to those areas of the skin which have been exposed to the sun light. The doses should be adjusted to achieve inhibition of cytokine release as a result of DNA damage. This means that for short periods of time surrounding the genotoxic exposure the dosage level of one or more DNA-protein kinase inhibitors, such as rapamycin, will be higher than at other times during transplant rejection therapy.

In some cases, in accordance with the invention, one or more transplant rejection drugs which are DNA-protein kinase inhibitors, e.g., rapamycin and its analogs (such as SDZ RAD), are used in conjunction with one or more transplant rejection drugs which are not such inhibitors, e.g., cyclosporin A or ascomycin. Since drugs of these two types generally do not have overlapping toxicities, this combination allows for greater flexibility in achieving the goal of immunosuppression, while at the same time, allowing

for protection of the patient from cytokine production as a result of exposure to one or more genotoxic agents.

In accordance with these aspects of the invention, the transplant rejection drug or drugs which are DNA-protein kinase inhibitors are used at a level which contributes at least some immunosuppression, but more importantly, are used at a level and/or in a manner which inhibits cytokine release in response to genotoxic agents. The relative amounts of the two types of immunosuppressive drugs are determined for each patient based on the patient's sensitivities to the drugs and on the amount of DNA-protein kinase inhibitor(s) required to achieve a desired level of protection from cytokine release.

Although the most common application of the invention is in connection with the reduction of cytokine production, in some cases, the invention can be used to enhance cytokine production in response to genotoxic agents. In particular, the invention can be used in the treatment of a variety of diseases, including autoimmune diseases, which respond to immunosuppressive genotoxic agents.

For example, enhanced cytokine production can be used in the treatment of psoriasis, a skin disease characterized by keratinocyte hyperproliferation and T-cell infiltration into the skin. Two common genotoxic agents used to treat this disease are coal tar and psoralen-plus-light. In each case, the genotoxic treatment causes cytokines to be released which suppress the T-cell activation and thus alleviate the disease symptoms. In accordance with the invention, DNA-protein kinase enhancers are used to increase the level of cytokine release in response to the genotoxic agent.

In particular, one or more DNA-protein kinase enhancers are administered to the patient just before or, preferably, at the time of administration of the immunosuppressive genotoxic agent(s). The use of these enhancers can permit reduction in the amount of genotoxic agent which needs to be administered and, in some cases, the enhancer(s) alone or

with only a minor amount of genotoxic agent can achieve the desired release of immunosuppresive cytokines.

Other diseases which can be addressed in this manner include atopic dermatitis, lupus erythematosus, arthritis, and porphyria. Each of these diseases produces inflammation through T-cell activation. Such T-cell activation can be suppressed by the appropriate immunosuppressive cytokines, and the DNA-protein kinase enhancers of the invention serve to augment the formation of those immunosuppressive cytokines upon exposure to genotoxic agents.

F. Assays for Levels of DNA-Protein Kinase Activity

The discovery that DNA-protein kinases are a central biological link between genotoxic agents and cytokine release allows those kinases to serve as measurement points (biological endpoints) for (1) sensitivity of individuals to genotoxic agents and (2) the effectiveness of modulators of cytokine release.

Thus, by measuring the level of a DNA-protein kinase activity of an individual, one can determine the level of sensitivity of that individual to genotoxic agent(s) which produce the type of DNA damage to which the DNA-protein kinase responds. For example, to measure the sensitivity to UV, one would measure the level of FRAP activity, while to measure the sensitivity to ionizing radiation (x-rays), one would measure the level of ATM activity. A high measured level of DNA-protein kinase activity indicates an individual who will respond to the genotoxic agent(s) with a high level of cytokine release, and a low measured level indicates an individual who will respond to the agent(s) with low levels of cytokine release.

Either case is undesirable and its identification allows suitable diagnostic and/or therapeutic procedures to be undertaken. In particular, for individuals with high levels of a particular DNA-protein kinase activity, an inhibitor or inhibitors for that DNA-protein kinase can be used as described above to modulate the individual's cytokine response to the

genotoxic agent(s) associated with that kinase. For individuals with low levels of DNA-protein kinase activity, further diagnostic procedures can be undertaken to determine the source of the low level of activity, e.g., a genetic screening can be performed. Such a DNA-protein kinase assay can provide a basis for undertaking a screening which otherwise would not be conducted.

With regard to the effectiveness of modulators of cytokine release, by conducting an assay for DNA-protein kinase activity, one can determine if a sufficient amount of a DNA-protein kinase inhibitor or enhancer has been administered to a patient and adjustments in the dose, mode of delivery, or delivery schedule can be made as appropriate.

In the case of patients undergoing immunosuppressive therapy using an immunosuppressive agent which is a DNA-protein kinase inhibitor, e.g., rapamycin, assays for DNA-protein kinase activity can be used to obtain a desired level of the immunosuppressive agent. In particular, because of the complex metabolism and biodistribution of immunosuppressive drugs such as rapamycin, blood levels of the parent compound may not be representative of the biological effectiveness of the parent compound and its metabolites. An assay for DNA-protein kinase activity more directly monitors the biological effectiveness of the compound and any of its metabolites.

Assays for levels of DNA-protein kinase activity which can be used in the practice of the invention include those which employ a radiolabeled ATP substrate, e.g., ^{32}P-ATP, a peptide substrate, gel electrophoresis, and an autoradiographic readout. See, for example, D. Price, J. Grove, V. Calvo, J. Avruch and B. Bierer, "Rapamycin-Induced Inhibition of the 70-Kilodalton S6 Protein Kinase," Science, volume 257, pages 973-977, 1992.

A preferred assay which avoids the use of radiolabeled reactants and which can simultaneously process many more samples than the Price et al. procedure is illustrated in Example 4 below. When used to determine a DNA-protein kinase level of a subject, the assay comprises the steps of: (1)

a sample of cells is obtained from the subject, (2) a preparation containing DNA-protein kinase(s) is obtained from the sample using anti-DNA-protein kinase antibodies, (3) the preparation is exposed to DNA damage of the type(s) the kinase(s) is (are) sensitive to together with the appropriate substrate(s) for the kinase(s), and (4) the level of phosphorylation of the substrate(s) is used as a measure of the level/activity of the kinase(s). The level of substrate phosphorylation is quantified using standard ELISA methods with antibodies specific to the substrate when phosphorylated by the DNA-protein kinase under assay.

The specificity and sensitivity of the assay can be modified by changing or combining the types and/or concentrations of the anti-DNA-protein kinase antibodies and/or the substrates used in performing the assay. In particular, levels of DNA-protein kinase activity for a plurality of kinases can be determined simultaneously by forming mixtures of anti-DNA-protein kinase antibodies and substrates in steps (2) and (3), respectively. The sensitivity of the assay can be modified by increasing antibody and/or substrate concentrations and/or reaction times.

G. Treatment Compositions

The DNA-protein kinase inhibitors of the invention may be formulated alone with suitable vehicles or they can be combined with each other and/or with other pharmaceutical ingredients, e.g., genoprotective agents which are not DNA-protein kinase inhibitors. One such example is a combination of rapamycin with one or more sunscreens, e.g., titanium dioxide, and/or one or more DNA repair enzyme(s), e.g., T4 endonuclease V, in topical formulations, to be used prior to, during or after exposure to one or more genotoxic agents, such as solar UV. In the case of DNA repair enzymes, encapsulation of the DNA repair enzyme in liposomes is a preferred method of administration. See Yarosh, U.S. Patents Nos. 5,077,211, 5,296,231, and 5,272,079.

The levels of DNA-protein kinase inhibitors in such formulations are selected as described above, e.g., using a DNA-protein kinase activity assay.

The levels of the other active ingredients in the formulation will generally correspond to the levels of the ingredient when used by itself.

In the case of patients undergoing chemotherapy, the DNA-protein kinase inhibitors of the invention, e.g., wortmannin, can be combined with a genotoxic chemotherapy agent, e.g., mitomycin C, for contemporaneous administration.

The DNA-protein kinase enhancers of the invention may also be formulated alone with suitable vehicles or they can be combined with each other and/or with pharmaceutical agents which are not DNA-protein kinase enhancers, e.g., with genotoxic agents. One example is a combination of damaged DNA, psoralen, and a suitable vehicle for application to the skin of a psoriasis patient.

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The materials and methods which are common to the examples are as follows.

MATERIALS AND METHODS

Cell Culture

The human immortalized HaCat cell line was from Dr. Jonathan Garlick, State University of New York at Stony Brook. The XPTNF2 cell line was prepared by transfection of the XP group A SV-40-transformed fibroblast cell line XP12BE with $pCAT_{TNF}$, as described by J. Kibitel, V. Hejmadi, L. Alas, A. O'Connor, B. Sutherland and D. Yarosh in "UV-DNA Damage in mouse and human cells induces the expression of tumor necrosis factor α", Photochemistry and Photobiology, volume 67, pages 541-546, 1998. This cell line expresses the chloramphenicol acetyltransferase gene from the mouse TNFα promoter. These transformed cell lines were grown in Dulbecco's modified Eagle's medium with 10% newborn calf serum and antibiotics at 37°C in a humidified, 5% CO_2 incubator. Non-transformed human keratinocytes were purchased from Clonetics Corporation, San

Diego, California, and grown in the protein-free keratinocyte growth media supplied with the cells, at 37°C in a humidified, 5% CO_2 incubator.

Drugs and genotoxic treatment

Rapamycin, wortmannin, staurosporine and lipopolysaccharide (LPS) were from Sigma Chemical Company. The drugs were prepared at 1,000-fold concentration and diluted into media just before use. Rapamycin-, wortmannin- or staurosporine- treated cells were pretreated for 30 minutes before UV irradiation, and for 18 hours after irradiation. For LPS treatment, the LPS was added to the cells for one hour at 37°C, and then the cells were refed with fresh media for 18 hours.

The UV was delivered from a Westinghouse FS-40 unfiltered sunlamp at 3.2 J/m²/sec. For UV irradiation, the media was removed, and the cells were irradiated and then refed with the same media for 18 hours.

Cell extracts and Western blots

Normal human epidermal keratinocytes were grown to 90% confluence in 10-cm plates, and then treated with drugs and genotoxic agents. After incubation, the cells were collected with a standard running buffer containing sodium dodecyl sulfate (SDS), sonicated for 2 seconds with a Heat Systems Ultrasonic Sonicator at 70% power, boiled in water for 5 minutes and stored at -70°C.

For Western blots, 10 μg of cell extracts were mixed with 5 μl of sample buffer containing running dye and the mixture boiled for 2 minutes and loaded into a standard SDS-PAGE gel with a stacking buffer (Biorad mini Protean II apparatus). For the $p70^{S6K}$ Western, a 10% SDS-PAGE was used, and for the TNFα Western, a 15% SDS-PAGE was used. The gels were run at 25 mAmps per gel until the running dye reached the bottom of the gel. The proteins were transferred to Immobilon P membrane by

electrophoretic transblotting in a Semi-phor apparatus (Hoefer Scientific Instruments, San Francisco, California) at 0.83 mAmps/cm^2 for 45 min. The blots were blocked with 5% non-fat dry milk, and probed with primary antibody at 4°C overnight. For the p70^{S6K} blot the antibody was specific for the form phosphorylated at threonine-421 and serine-424 (New England Biolabs). The blot was then washed and incubated with horse-radish peroxidase linked anti-rabbit antibody. The blot was developed with the ECL kit (Amersham) using Hyperfilm ECL for exposure. For the TNFα blot the monoclonal antibody against human TNFα was from Boehringer Mannheim Biochemicals. The blots were then washed and incubated with goat anti-mouse IgG linked to biotin and then avidin-horse radish peroxidase, and then developed with the ECL kit as above.

TNFcat assay

The CAT assays were performed as described in Kibitel et al., 1998. Briefly, the XPTNF2 cells were treated with drugs and genotoxic agents and incubated for 18-24 hours. Extracts were prepared by three rounds of freezing and thawing and centrifugation, and 50 μg of each extract was mixed with 5 nmoles of BODIPY-chlorampenicol (Molecular Probes, Eugene, Oregon) and 0.5mM acetyl-coA (Sigma Chemical Company). After 30 minutes incubation at 37°C, the reaction products were extracted with cold ethyl acetate, and analyzed by thin layer chromatography. The fluorescent substrate and the acetylation products were visualized by UV-A, and the digitized image was analyzed by computerized image analysis to calculate the fraction of acetylated chloramphenicol and thus the CAT activity.

Immunoprecipitation and DNA protein kinase activity assay

HaCat cells were grown in 10-cm dishes to near confluence. They were then sonicated for 10 sec with the Heat Systems Ultrasonicator microtip at 70% power in TGN Buffer (50 mM Tris, pH 7.5, 50 mM glycerophosphate,

150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM DTT, 0.5 µl/ml Sigma P8340 protease inhibitor cocktail, 2 nM microclystin LR). After centrifugation, the extract was adjusted to 1 mg/ml protein with the same buffer and stored at -70°C. To assay DNA protein kinase activity, 1 mg of HaCat extract was incubated at 4°C for 2 hours with 2 µg of goat anti-FRAP or anti-ATM antibody (Santa Cruz Biotechnology), then mixed with 40 µl of Protein G PLUS-agarose (Santa Cruz Biotechnology) and rolled at 4°C overnight.

The agarose beads were collected by centrifugation, washed with TGN buffer, then high salt buffer (100 mM Tris, pH 7.4, 500 mM LiCl), then PK-buffer (25 mM HEPES-KOH, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 µl/ml Sigma P8340 protease inhibitor cocktail, 2 nM microclystin LR, 200 µM ATP), and resuspended in 50 µl PK- buffer. Modifiers of the reaction included 0.5 µg λ-DNA or λ-DNA irradiated with 250 J/m² UV-C from a G15T germicidal lamp, and rapamycin at 40 ng/ml. To initiate the reaction, 2.6 µg FKBP (Sigma Chemical Company) and was added to the FRAP reactions, and then 50 µg p53 peptide (amino acids 1-393, Santa Cruz Biotechnology) and 1 nmol ATP were added to all the DNA protein kinase reactions. After 2 hours incubation at 30°C, the reaction products were separated from the agarose beads by centrifugation.

The reaction products were diluted 8-fold into standard ELISA Coating Buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, 0.1 g/L thimerisol), and 200 µl were placed in wells of a Immulon 2HB 96-well ELISA plate (Dynex Technologies Inc., Chantilly, Virginia), and incubated at 4°C, overnight. The wells were washed and blocked with 5% bovine serum albumin for 60 minutes at room temperature. The wells were then incubated with a mixture of 2.5 µl anti-phosphoserine-BSA antibody linked to biotin and 2.5 µl antiphosphothreonine-BSA antibody linked to biotin (Sigma Chemical

Company) in 10 ml TBS/NonI (25 ml/L 2M Tris pH 8, 30 ml/L 5 M NaCl, 0.1% Nonidet P-40) for 60 minutes at room temperature. The wells were washed and incubated with 100 µl of a mixture of 10 µl avadin-alkaline phosphatase (Sigma Chemical Company) in 10 ml TBS/NonI for 60 minutes at room temperature. The plates were washed, developed with phospho-nitrophenylphosphate in 0.1 M diethanolamine pH 10, and read at 405_{nm} by a microtiter plate reader.

Example 1

This example demonstrates that TNFα is expressed by normal human keratinocytes after UV exposure and that this expression is inhibited by rapamycin.

Human keratinocytes from the HaCat cell line were irradiated with 200 J/m² UV-B from an FS40 sunlamp. Parallel cultures were treated with rapamycin at 2 ng/ml for 30 minutes prior to and then 60 minutes after irradiation. After 24 hours incubation at 37°C, extracts were prepared from these cells by scraping them into a phosphate-saline buffer and sonicating them with a 2 sec exposure to the microtip of an ultrasonicator (Heat Systems Ultrasonicator) at 70% maximum energy.

Ten micrograms of protein were then loaded in each well of a 15% polyacrylamide gel and electrophoresed. The separated proteins were eluted onto nitrocellulose filter paper by semi-dry transblotting and probed with antibody against human TNFα. A sample of human TNFα served as a standard.

As shown in Figure 4, UV induces TNFα, as can be seen in lane 1. Rapamycin at 2 ng/ml inhibited the expression of TNFα, as seen by the great reduction in this band in lane 2. In contrast, lipopolysaccharide (LPS) induces TNFα without DNA damage by binding to the cell surface membrane receptor CD14. Cells treated with 1 µg/ml LPS induced TNFα (see lane 3) and rapamycin had no effect on this LPS induction of TNFα (compare lane 3 to lane 4).

Example 2

This example demonstrates that the induction of TNFα mRNA expression by UV is inhibited by rapamycin.

To illustrate this principle, human cells were used that carried a transgene composed of the chloramphenicol acetyltransferase (CAT) gene under the control of the tumor necrosis factor α (TNFα) promoter. This system has been used to investigate those stimuli that cause transcription of the TNFα gene. Transcription of the CAT gene is readily measured by a simple enzymatic assay that measures the formation of acetylated forms of chloramphenicol by thin layer chromatography. Examples of these assays are shown in Figure 5.

Substrate alone is shown in lane 1 and background levels of acetylation by untreated cell extracts is shown in lanes 2, 6 and 9. CAT expression from the TNFα promoter is reflected by increasing acetylation of the fluorescent chloramphenicol substrate, resulting in faster migrating species (from bottom to top) in the thin layer chromatography assay.

TNFα is induced by genotoxic treatments, such as UV (lanes 3,7 and 10), and by non-genotoxic treatments, such as treatment with LPS (lane 12). UV induction of TNFα is inhibited by rapamycin (lane 5), demonstrating that the DNA-protein kinase FRAP is required for transduction of the signal of DNA damage into expression of the cytokine TNFα gene.

Rapamycin alone has no effect compared to untreated cells (compare lane 4 with lane 2). TNFα induction is also inhibited by wortmannin at 500 nM, a dose that inhibits DNA-protein kinases (lane 8), and also by staurosporine at 200 nM (lane 11), a dose that specifically inhibits serine phosphorylation, a characteristic type of phosphorylation by DNA-protein kinases. These results clearly show that UV induction of expression from the TNFα promoter requires DNA-protein kinases in general, and FRAP kinase in specific, and involves phosphorylation at serine resides.

In contrast, induction of TNFα by the non-genotoxic agent LPS was not inhibited by rapamycin as shown in lanes 12 and 13 and in Figure 6.

In sum, this example demonstrates that the pathway leading from UV-DNA damage to TNFα transcription requires rapamycin-sensitive DNA-protein kinases, and that non-DNA damage events at the cell membrane or elsewhere do not involve such kinases.

Example 3

As known in the art, downstream in the pathway following activation of the FRAP kinase is phosphorylation of the p70S6K kinase, which phosphorylates ribosomal proteins and alters translation of gene transcripts. One measure of UV-specific activation of the FRAP kinase is thus phosphorylation of p70S6K. This example uses this measure to demonstrate such activation.

Human keratinocytes were pre-treated with 2 ng/ml rapamycin, then treated with UV-irradiation or LPS and extracted as described above (see Example 1 and Materials and Methods). The extracts were electrophoresed in a 10% polyacrylamide gel and then probed with antibodies specific for the serine/threonine phosphorylated forms of p70S6K in a Western blot. As a loading control, the polyacrylamide gel was stained with Coomassie blue to identify total protein loaded in each lane.

As shown in Figure 7, UV irradiation increased phosphorylation of $p70^{S6K}$ (compare lanes 1 and 2) and this phosphorylation was blocked by pre-treatment with rapamycin (lane 3). In contrast, the LPS induced phosphorylation of p70S6K (lane 4) was comparatively insensitive to rapamycin (compare lanes 4 and 5). The loading control bands shown at the bottom of this figure demonstrate the equivalent loading of protein in the gel.

Example 4

This example demonstrates the direct activation of DNA-protein kinase activity by damaged or broken DNA.

ATM or FRAP in extracts of the human keratinocyte cell line HaCat were immunoprecipitated by incubation with (1) antibodies against either ATM or FRAP and (2) immunoprecipitating anti-antibodies linked to agarose beads.

The bound ATM or FRAP was collected by centrifugation, and then mixed with a phosphorylation substrate polypeptide derived from the p53 protein, and in the case of FRAP, with its small subunit protein FKBP. In some cases bacteriophage λ DNA, UV-irradiated DNA, or rapamycin were added to the reactions. Adenosine triphosphate was then added and the reactants incubated for 2 hours at 30°C. The reaction products were then bound to an ELISA plate and probed with antibodies specific for phosphoserine and phosphothreonine. The binding of these antibodies was detected by (1) secondary antibodies linked to alkaline phosphatase and (2) nitrophenyl phosphate substrate. The resulting yellow color was measured by optical density at 405 nm using a multiwell plate reader.

The results are shown in Figure 8. As shown therein, FRAP phosphorylation of the p53 peptide was stimulated by the addition of λ DNA, whose short size resembled mammalian DNA with many broken ends (compare the FRAP/FKBP/p53 bar with the +DNA bar). However, the FRAP activity was much greater when the DNA that was added was first irradiated with UV to induce photoproducts (compare the +DNA bar with the +UV-DNA bar). This UV-DNA enhanced phosphorylation was completely abrogated by coincubation with 2 ng/ml rapamycin (+rapamycin bar). Similarly, ATM phosphorylated the p53 peptide (ATM/p53 bar), and its phosphorylation was stimulated by addition of λ DNA (+DNA bar), whose short size resembles broken DNA.

Example 5

This example shows that rapamycin's ability to reduce induction of TNFα by UV is dose dependent.

XPTNF2 cells irradiated with 100 J/m² UV-B to induce expression of the CAT gene from the TNFα promoter were incubated with increasing

concentrations of rapamycin. The levels of inhibition of expression from the promoter are shown in Figure 9.

As can be seen therein, at levels of rapamycin of less than 2 ng/ml, the inhibitory effect of this DNA-protein kinase inhibitor was undetectable. The effect became detectable at 2 ng/ml, and higher doses were increasingly inhibitory.

Although specific embodiments of the invention have been described and illustrated, it is to be understood that modifications can be made without departing from the invention's spirit and scope.

The contents of the various literature citations referred to above are hereby incorporated herein by reference.

What is claimed is:

1. A method of treating a human to inhibit cytokine release resulting from exposure to a genotoxic agent, said method comprising administering a DNA-protein kinase inhibitor to said human in an amount sufficient to inhibit cytokine release by cells exposed to the genotoxic agent.

2. The method of Claim 1 wherein the genotoxic agent is ultraviolet light.

3. The method of Claim 1 wherein the genotoxic agent is a chemotherapy agent or a radiotherapy agent.

4. The method of Claim 1 wherein the DNA-protein kinase inhibitor is rapamycin.

5. The method of Claim 1 wherein the DNA-protein kinase inhibitor is administered in an amount sufficient to inhibit release of at least one cytokine selected from the group consisting of interleukin-1, interleukin-6, tumor necrosis factor α, interleukin-10, and intercellular adhesion molecule 1.

6. A method of treating a human to inhibit cytokine release resulting from exposure to a genotoxic agent, said method comprising administering a DNA-protein kinase inhibitor to said human in an amount sufficient to reduce the activity of at least one DNA-protein kinase to thereby inhibit cytokine release by cells exposed to the genotoxic agent.

7. The method of Claim 6 wherein the genotoxic agent is ultraviolet light.

8. The method of Claim 6 wherein the genotoxic agent is a chemotherapy agent or a radiotherapy agent.

9. The method of Claim 6 wherein the DNA-protein kinase inhibitor is rapamycin.

10. The method of Claim 6 wherein the DNA-protein kinase inhibitor is administered in an amount sufficient to reduce the activity of at least one DNA-protein kinase selected from the group consisting of DNA-PK, ATM, ATR, and FRAP.

11. The method of Claim 6 wherein the DNA-protein kinase inhibitor inhibits release of at least one cytokine selected from the group consisting of interleukin-1, interleukin-6, tumor necrosis factor α, interleukin-10, and intercellular adhesion molecule 1.

12. A method for modifying the effects of a genotoxic agent comprising modifying the release of cytokines resulting from the DNA damaging effects of the genotoxic agent by modulating DNA-protein kinase activity.

13. A method for treating a side-effect of a genotoxic agent comprising reducing the release of cytokines by modulating DNA-protein kinase activity while preserving the DNA damaging effects of the genotoxic agent.

14. The method of Claim 13 wherein the side-effect is selected from the group consisting of skin cancer, erythema, viral activation, inflammation, fever, nausea, vomiting, headaches, chills, abnormal pigmentation, alopecia, and combinations thereof.

15. A method for reducing at least one side-effect of transplant rejection therapy for a patient undergoing such therapy comprising:

(a) administering a compound which inhibits transplant rejection at a first dosage level; and

(b) administering an additional amount of said compound in connection with exposure of the patient to a genotoxic agent, said compound being a DNA-protein kinase inhibitor.

16. The method of Claim 15 wherein the compound is rapamycin.

17. A method for reducing at least one side-effect of transplant rejection therapy for a patient undergoing such therapy comprising:

(a) administering a first compound which is capable of inhibiting transplant rejection to the patient; and

(b) administering a second compound which is capable of inhibiting transplant rejection to the patient in connection with exposure of

the patient to a genotoxic agent, said second compound being a DNA-protein kinase inhibitor.

18. The method of Claim 17 wherein the first compound is cyclosporin-A.

19. The method of Claim 17 wherein the second compound is rapamycin.

20. A method for conducting transplant rejection therapy comprising administering a compound which is capable of inhibiting transplant rejection to a patient in need of such therapy in an amount sufficient to inhibit cytokine release as result of exposure to a genotoxic agent, said compound being a DNA-protein kinase inhibitor.

21. The method of Claim 20 wherein the compound is rapamycin.

22. A method for conducting transplant rejection therapy comprising administering a compound to a patient in need of such therapy in an amount sufficient to both control transplant rejection and inhibit cytokine release as result of exposure to a genotoxic agent, said compound being a DNA-protein kinase inhibitor.

23. The method of Claim 22 wherein the compound is rapamycin.

24. A method for conducting transplant rejection therapy comprising administering a first compound to a patient in need of such therapy in an amount sufficient to contribute to the control of transplant rejection and administering a second compound to the patient in an amount sufficient to both contribute to the control of transplant rejection and to inhibit cytokine release as result of exposure to a genotoxic agent, said second compound being a DNA-protein kinase inhibitor.

25. The method of Claim 24 wherein the first compound is cyclosporin-A.

26. The method of Claim 24 wherein the second compound is rapamycin.

27. A method for enhancing cytokine release comprising: (a) administering at least one genotoxic agent to a tissue or organ of a human,

and (b) administering at least one DNA-protein kinase enhancer to said tissue or organ in an amount sufficient to enhance release of cytokines by the tissue or organ in response to the genotoxic agent.

28. The method of Claim 27 wherein the tissue or organ is suffering from an inflammatory disease.

29. The method of Claim 28 wherein the tissue or organ is skin, the inflammatory disease is psoriasis, and the genotoxic agent is selected from the group consisting of coal tar and psoralen-plus-light.

30. A method for monitoring the administration of an immunosuppressive agent to a patient, said immunosuppressive agent being an inhibitor of at least one DNA-protein kinase, said method comprising:

(a) obtaining a sample of cells from the patient; and

(b) determining an activity level for said at least one DNA-protein kinase for said sample.

31. The method of Claim 30 comprising the further step of adjusting at least one of the amount, schedule, or route of delivery of the immunosuppressive agent based on the determination of step (b).

32. The method of Claim 30 wherein the immunosuppressive agent is rapamycin.

33. A method for determining sensitivity of an individual to one or more genotoxic agents comprising:

(a) obtaining a sample of cells from the individual; and

(b) determining for said sample an activity level for at least one DNA-protein kinase.

34. A method for determining sensitivity of an individual to a genotoxic agent comprising:

(a) obtaining a sample of cells from the individual; and

(b) determining for said sample an activity level for a DNA-protein kinase which is specific for DNA damage of the type produced by said genotoxic agent.

35. A method for determining activity of a DNA-protein kinase in a biological sample comprising:

(a) isolating the DNA-protein kinase from the sample;

(b) exposing the isolated kinase to DNA damage of the type the kinase is sensitive to together with a substrate which the kinase can phosphorylate; and

(c) determining the level of substrate phosphorylation, said level being indicative of the activity of the kinase.

36. The method of Claim 35 wherein the level of substrate phosphorylation is determined using an antibody specific to the substrate when phosphorylated.

37. A composition comprising a DNA-protein kinase inhibitor and a genotoxic agent.

38. The composition of Claim 37 wherein the genotoxic agent is a chemotherapy agent.

39. The composition of Claim 37 wherein the genotoxic agent is coal tar.

40. A composition comprising a DNA-protein kinase inhibitor and psoralen.

41. A composition comprising a DNA-protein kinase inhibitor and immunosuppressive agent which is not a DNA-protein kinase inhibitor.

42. The composition of Claim 41 wherein the DNA-protein kinase inhibitor is rapamycin and the immunosuppressive agent which is not a DNA-protein kinase inhibitor is cyclosporin-A.

43. A composition comprising a DNA-protein kinase inhibitor and a genoprotective agent which is not a DNA-protein kinase inhibitor.

44. The composition of Claim 43 wherein the genoprotective agent which is not a DNA-protein kinase inhibitor is a sunscreen.

45. The composition of Claim 43 wherein the genoprotective agent which is not a DNA-protein kinase inhibitor is a DNA repair enzyme.

46. The composition of Claim 43 wherein the DNA-protein kinase inhibitor is rapamycin.

47. A method of reducing a side-effect of administering or delivering chemotherapy or radiotherapy to a patient comprising administering a DNA-protein kinase inhibitor to at least one of the patient's gastrointestinal track, scalp, or a location at which the chemotherapy or radiotherapy is administered or delivered.

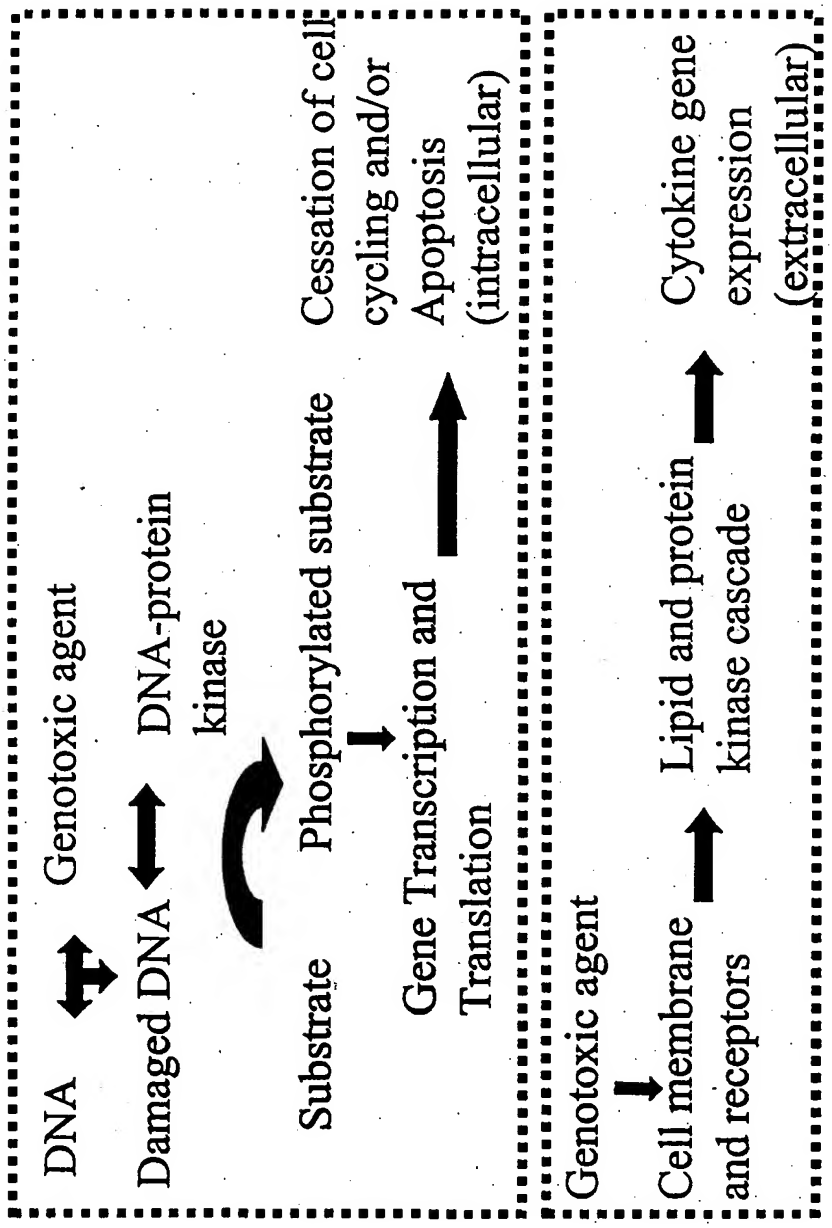
PRIOR ART
DNA
Genotoxic agent
Damaged DNA
DNA-protein kinase
Substrate
Phosphorylated substrate
Gene Transcription and Translation
Cessation of cell cycling and/or Apoptosis (intracellular)
Genotoxic agent
Cell membrane and receptors
Lipid and protein kinase cascade
Cytokine gene expression (extracellular)

FIG. 1

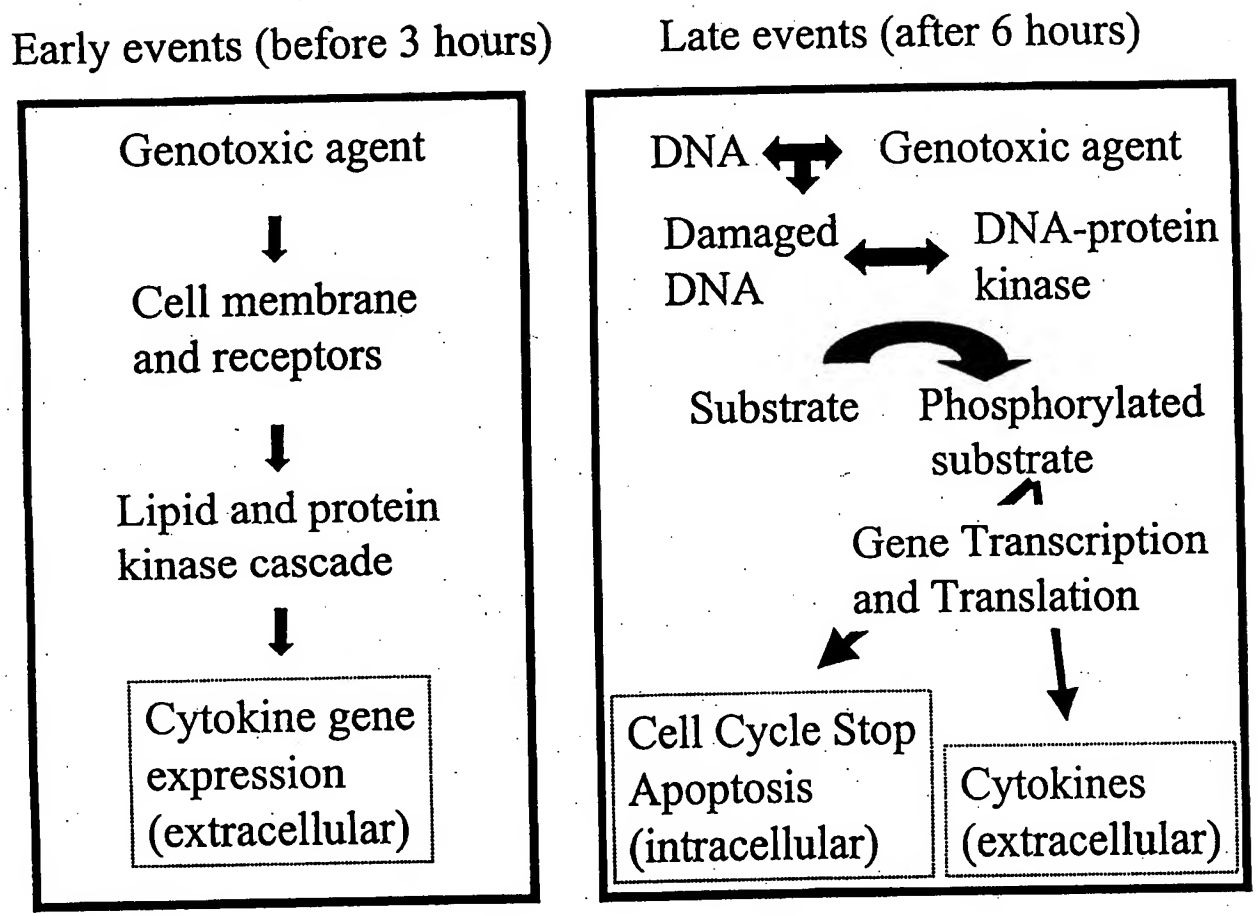
Early events (before 3 hours)
Genotoxic agent
Cell membrane and receptors
Lipid and protein kinase cascade
Cytokine gene expression (extracellular)
Late events (after 6 hours)
DNA
Genotoxic agent
Damaged DNA
DNA-protein kinase
Substrate
Phosphorylated substrate
Gene Transcription and Translation
Cell Cycle Stop Apoptosis (intracellular)
Cytokines (extracellular)

FIG. 2

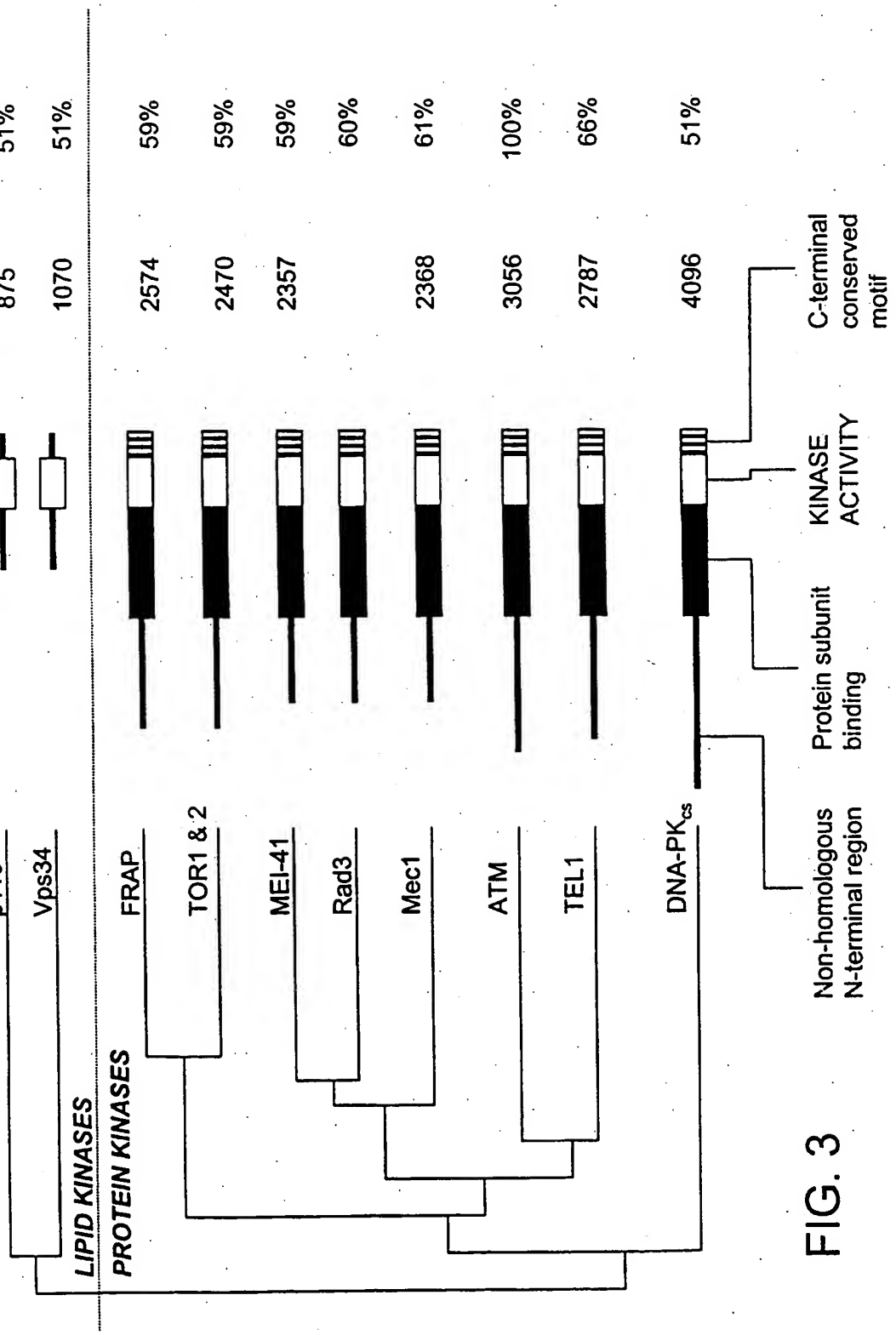
Kinase Name
Protein Organization
Number of Amino Acids
Kinase Similariy
p110
875
51%
Vps34
1070
51%
LIPID KINASES
PROTEIN KINASES
FRAP
2574
59%
TOR1 & 2
2470
59%
MEI-41
2357
59%
Rad3
60%
Mec1
2368
61%
ATM
3056
100%
TEL1
2787
66%
DNA-PKcs
4096
51%
Non-homologous N-terminal region
Protein subunit binding
KINASE ACTIVITY
C-terminal conserved motif

FIG. 3

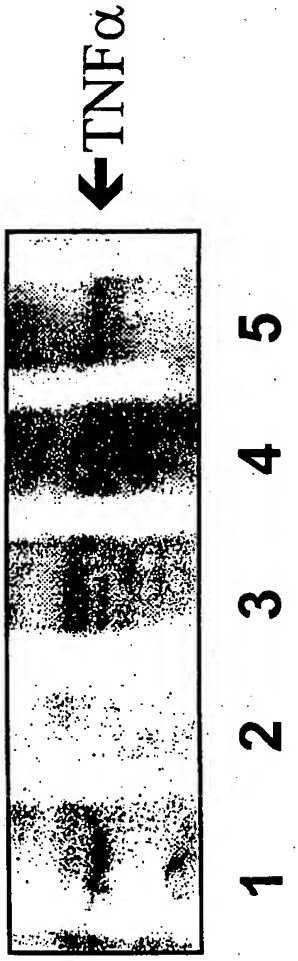

FIG. 4

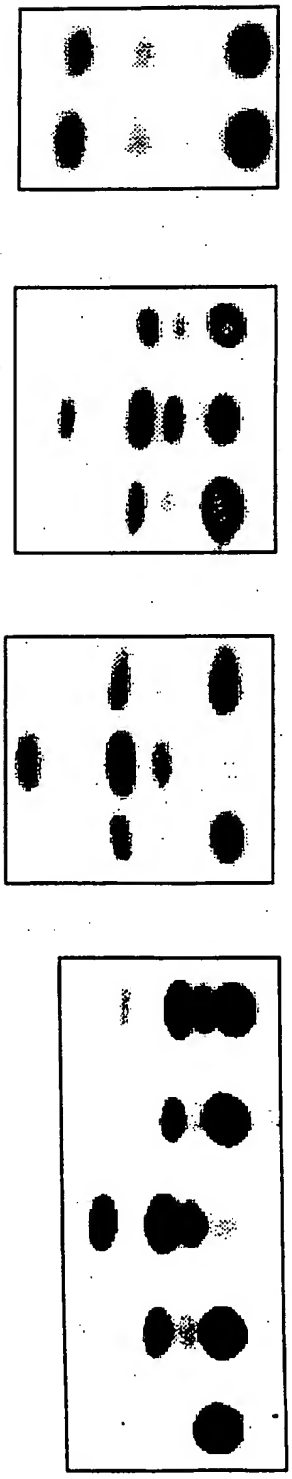

FIG. 5

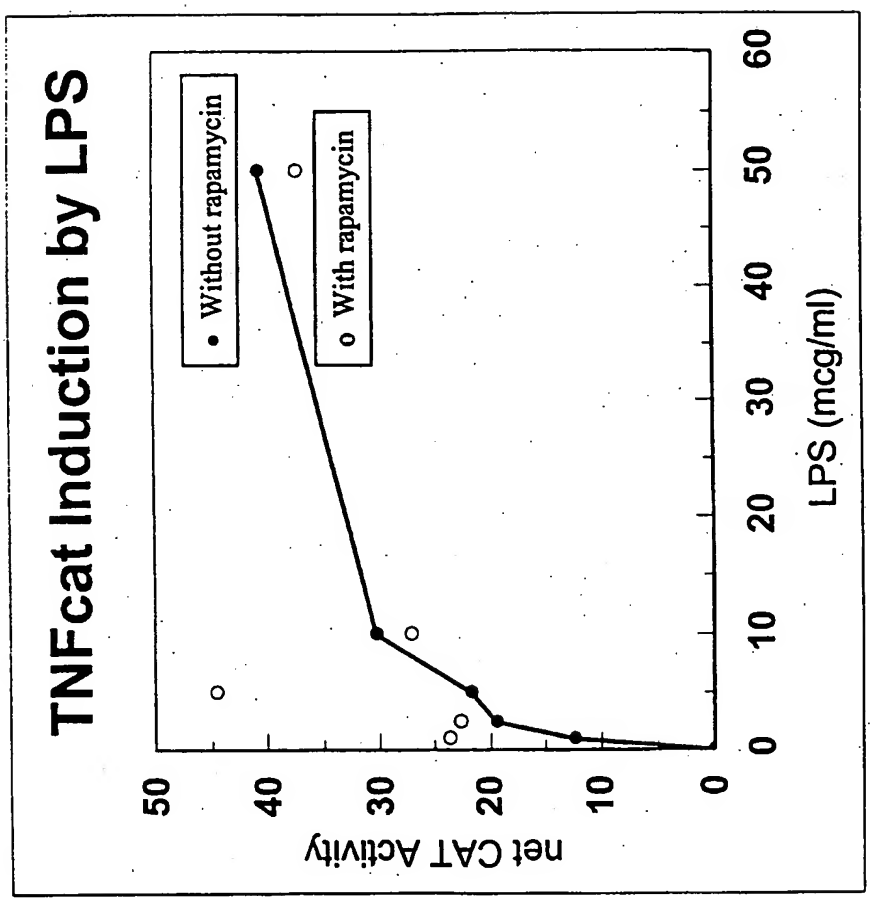
TNFcat Induction by LPS
• Without rapamycin
o With rapamycin
net CAT Activity
0
10
20
30
40
50
0
10
20
30
40
50
60
LPS (mcg/ml)

FIG. 6

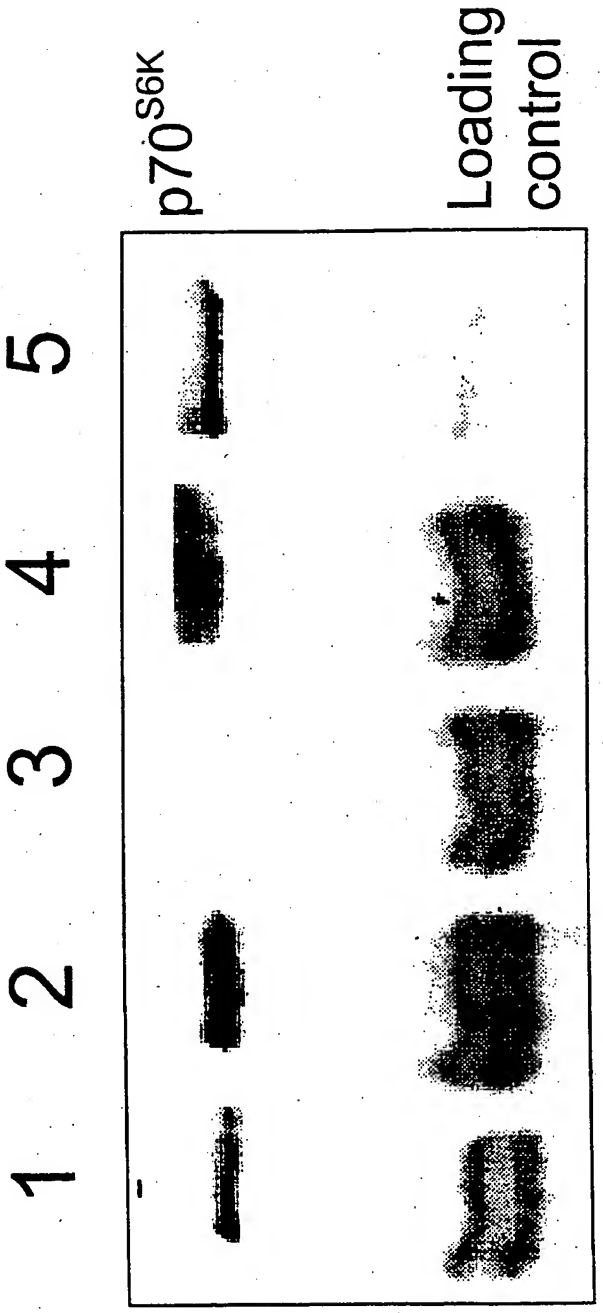

FIG. 7

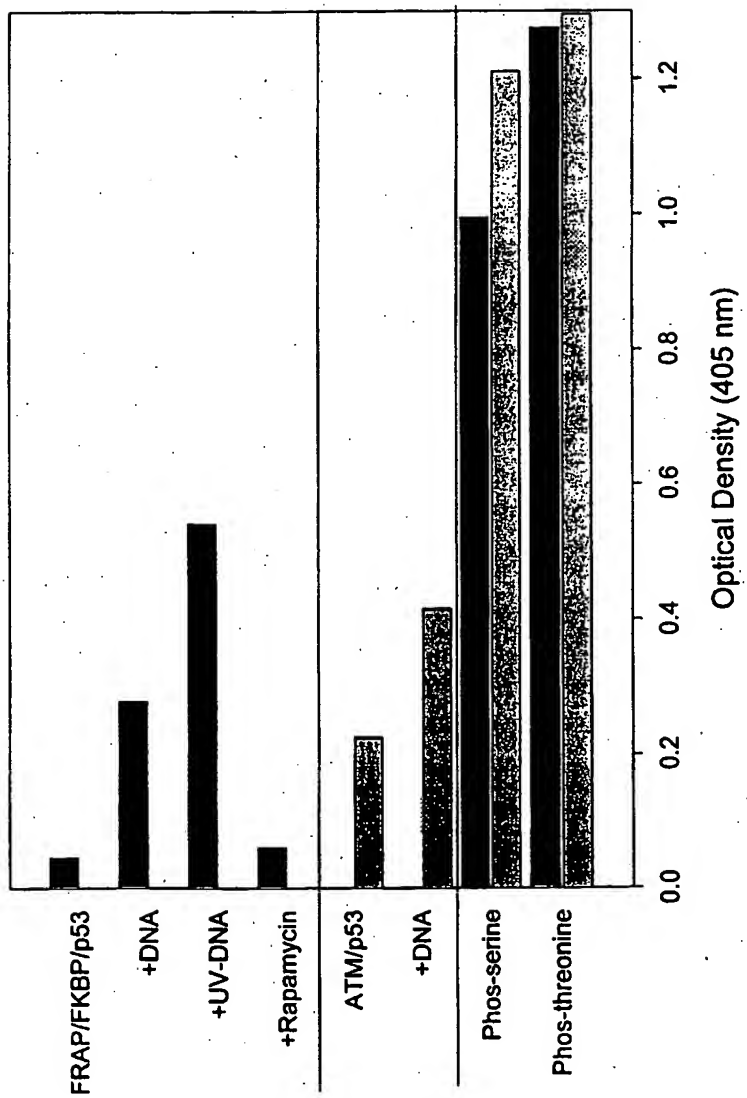
FRAP/FKBP/p53
+DNA
+UV-DNA
+Rapamycin
ATM/p53
+DNA
Phos-serine
Phos-threonine
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Optical Density (405 nm)

FIG. 8

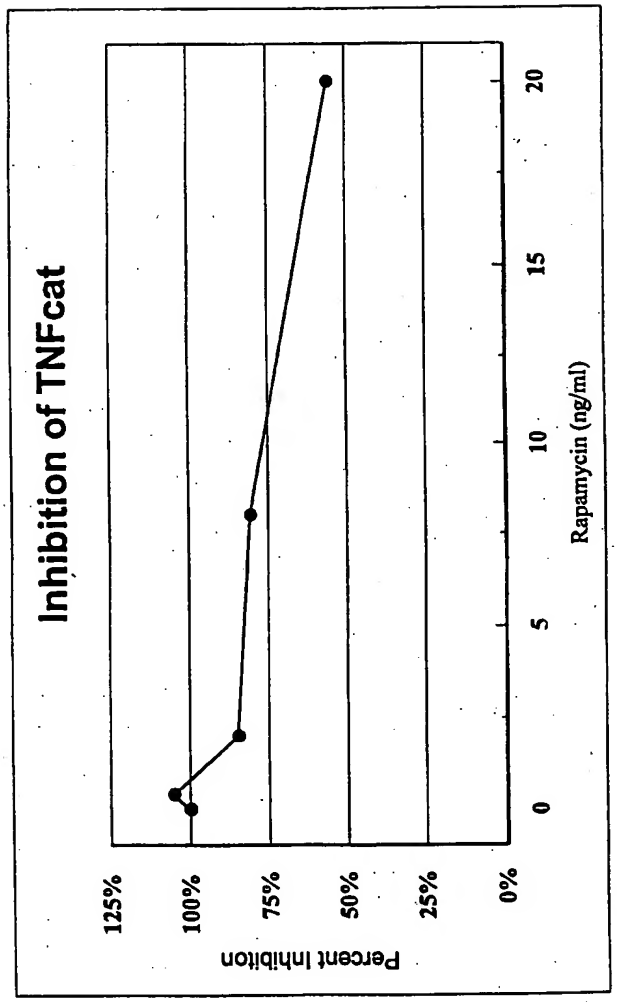
Inhibition of TNFcat
Percent Inhibition
125%
100%
75%
50%
25%
0%
0
5
10
15
20
Rapamycin (ng/ml)

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12348

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70, 7/40, 7/42, 38/43
US CL :514/25, 2, 8, 12, 885, 886, 887, 916

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/25, 2, 8, 12, 885, 886, 887, 916

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	HOSOI et al. A phosphatidylinositol 3-kinase inhibitor wortmannin induces radioresistant DNA synthesis and sensitizes cells to bleomycin and ionizing radiation. Int. J. Cancer. 1998, Vol. 78, pages 642-647.	1-47
A	LEES-MILLER, S.P. The DNA-dependent protein kinase, DNA-PK: 10 years and no ends in sight. Biochem. Cell Biol. 1996, Vol. 74, pages 503-512.	1-47
A	GLOVER et al. Skin cancer in renal transplant patients. Cancer Bull. 1993, Vol. 45, No. 3, pages 220-224.	15-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

14 APRIL 1999

Date of mailing of the international search report

20 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02348

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARIONNET et al. Differences in responses of interleukin-1 and tumor necrosis factor α production and secretion to cyclosporin-A and ultraviolet B-irradiation by normal and transformed keratinocyte cultures. Exp. Dermatol. 1997, Vol. 6, pages 22-28.	1-47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02348

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02348

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, CAPLUS, EMBASE, BIOSIS
search terms: cytokine, DNA-protein kinase inhibitor, genotoxic agent, chemotherapy agent, radiotherapy agent, ultraviolet light, rapamycin, cyclosporin-A, transplant rejection, administer, treat, therapy, monitor, sensitivity, activity, DNA repair enzyme

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-14, 37-42, 47, drawn to a method of treating a human with a DNA-protein kinase inhibitor, to inhibit cytokine release resulting from exposure to a genotoxic agent.
Group II, claims 15-26, drawn to a method for modifying the effects of a genotoxic agent comprising modifying the release of cytokines by modulating DNA-protein kinase activity.
Group III, claims 27-29, drawn to a method for enhancing cytokine release by administering at least one genotoxic agent and at least one DNA-protein kinase enhancer to enhance the release of cytokines.
Group IV, claims 30-34, drawn to a method for monitoring the administration of an immunosuppressive agent which is an inhibitor of a DNA-protein kinase.
Group V, claims 35-36, drawn to a method for determining activity of a DNA-protein kinase by determining the level of substrate phosphorylation.
Group VI, claims 43-46, drawn to a composition comprising a DNA-protein kinase inhibitor and a genoprotective agent which is not a DNA-protein kinase inhibitor.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first-recited process, a method of treating a human with a DNA-protein kinase inhibitor, to inhibit cytokine release resulting from exposure to a genotoxic agent. Further pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.